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Local and systemic factors affecting the outcome of endodontic treatment

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1 Introduction

We are living in the age of evidence-based medicine. Any new concept and technique to be used on patients should ideally be assessed in randomized controlled clinical trials against their respective gold standards. This, however, poses a major problem particularly in endodontic research. A favourable outcome of root canal treatment is defined as the reduction of a radiographic lesion and the absence of clinical symptoms of the affected tooth after a minimal observation period of 1 yr. (1). Alternatively, the so called surrogate outcome (dependent) variables yielding quicker results, such as the microbial load remaining in the root canal system after different treatment protocols, can be defined. However, these do not necessarily correlate with the "true" treatment outcome (2). Endodontic success is dependent on multiple factors (3), and a faulty treatment step can thus be compensated. For instance, if cultivable microbiota remain after improper canal disinfection, they can theoretically be entombed in the canal system by a perfect root canal filling (4), and clinical success may still be achieved (5). On the other hand, in a methodologically sound clinical trial, single treatment steps have to be randomized and related to outcome. Otherwise, the results do not allow any conclusions and no causative relationships may be revealed (6).

The above issues may be viewed as the reason (or as an excuse) for the fact that no randomized controlled clinical trials exist on the effect of irrigating solutions on treatment outcome in the endodontic literature. As of yet, we largely depend on data from in vitro studies and clinical trials with microbial recovery after treatment as the

surrogate outcome. Clinical recommendations based on such findings are merely deductive and need to be interpreted with care.

1.1 Root Canal Infection

A traditional concept that explains infectious processes occurring in humans suggests that diseases are produced as the result of the aggressive invasion of harmful microorganisms, which battle with the human host's defences, triggering mechanisms that release antibodies and immune cells. The impact of such an approach generates a predisposition to search for those "most dangerous" microorganisms that can cause/ trigger the most severe damage to the host. In line with this view, infectious processes of the oral cavity were proposed to be caused by a relatively small number of organisms from the diverse collection of species found in the human mouth (7). In caries, for example, the frequent isolation of *Streptococcus mutans* from carious lesions (8–12) generated a considerable number of studies to explore the ex vivo features of this bacterium. Research findings showing the significant acid-tolerant capabilities of *S. mutans* defined this organism as "the" agent responsible for initial enamel and dentine demineralization. Similarly, in periodontal disease, the frequent recovery of proteolytic microorganisms from deep periodontal pockets, such as *Porphyromonas gingivalis*, increased the attention of periodontists to these bacteria because they were considered key etiological agents of the disease (11,12). The main disadvantage with this traditional view of the infectious process, especially in oral infections, is that the determination of true cause-and-effect relationships is not always possible. Consequently, the predominance of certain microorganisms at a given site may be the result of the disease itself rather than that of the initiating agent (13). Recently, the "ecological plaque hypothesis" (14–19) has improved on these classic infectious concepts to explain the aetiology of caries and periodontal disease. This hypothesis suggests that the organisms associated with the disease may also be present at sound sites, but at levels too low to

represent a clinical threat. In other words, disease is produced as the result of changes in the local environmental conditions that will shift the balance of the resident flora.

Root canal infections have a different nature than that of caries or periodontitis because they become established in originally sterile compartments of the oral cavity. In many cases, this led to the concept that the aetiology of root canal infections involves only a single pathogen. For example, the predominance of certain proteolytic black-pigmented anaerobic organisms in cultures from infected root canals associated with acute symptoms suggested that these organisms are foremost etiological agents in such cases (20,21). Recently, the frequent recovery of Enterococcus faecalis in root canals associated with persistent infections brought about an intense research interest in this bacterium. E. faecalis has become the ideal organism to test different irrigants, medicaments, and antiseptic solutions used in endodontics ex vivo, with findings that revealed its innate resistance capacity (22–24). This extensive interest in E. faecalis, perhaps driven by its ability to grow under almost any laboratory condition (25), resulted in the concept that the organism is the sole etiological agent for chronic endodontic infections. Consequently, the focus on E. faecalis resulted in much less information on the existence of other organisms in such infections that may possess similar tolerating characteristics to E. faecalis and that would shed light on the existence of a polymicrobial persisting community. Thus, it is not surprising that ecological parameters in root canal infections are not often discussed.

From an ecological perspective, the root canal can be considered a highly controlled environment with a limited number of niches. Although niches are composed by a variety of environmental factors that limit the growth of one species relative to others (26), the main limiting factors in root canal niches that influence bacterial colonization are, for instance, oxygen and nutrient availability (27). After root canal treatment, other limiting factors become involved,

such as pH and the short/long-term effects of the antibacterial medicaments applied. Bacterial survival in such controlled environments, especially after root canal treatment, is based on the capacity of organisms to adapt to the existing conditions.

Although traditional views suggest that the organisms surviving root canal treatment are a selected group of the "most robust" organisms, the application of ecological parameters indicates that bacterial survival after root canal treatment will depend not on the robustness of the organisms, but on how good an adaptor the organism is to the new limiting factors in their corresponding niches. Furthermore, as in every natural microenvironment, the adaptive capabilities of individual organisms are exponentially augmented when growing in biofilm communities. The foundation for this ecological approach to endodontic infections suggests that the most dangerous "pathogen" is not an individual species, but a polymicrobial entity that undergoes physiological and genetic changes triggered by changes in the root canal environment.

Currently, there is no substantial evidence indicating that certain microorganisms of the microbial flora in root canal infections are more virulent than others. With this in mind, Sundqvist and Figdor (28) stated that a proper definition for endodontic pathogens should include every organism capable of inducing the tissue destruction in apical periodontitis. In reality, however, the majority of endodontic-microbiology studies refer to the endodontic pathogen as the bacterium isolated from a symptom-associated root canal that grows in the laboratory in a specific media. By this approach, the most frequently recovered species will assume the role of major endodontic pathogen. In persistent root canal infections, for example, the frequent occurrence of monocultures of E. faecalis has raised suspicion that this bacterium may be the sole organism persisting in the root canals. Considering that mono-infections rarely if ever occur in nature, it is possible that the apparent pure cultures of E. faecalis could be the result of sampling and culturing techniques that favour it over other

organisms at the site that were either in low numbers or were physiologically inactive or dormant. For instance, in a commonly cited study (29), from the total 100 root- filled teeth with apical periodontitis sampled E. faecalis was reported as the most frequently recovered organism (32%), although in 32% of the cases with persistent lesion no microbe could be isolated. In yet nine root-filled teeth without periapical lesion that showed bacterial growth, the organism was found in one case. In a similar study, 25 root-filled teeth requiring retreatment were sampled and E. faecalis was found in 14 of those 20 teeth with bacterial growth (30). However, it would seem that this study was focused primarily in proving the occurrence of E. faecalis in root-filled teeth rather than in exploring the microbial flora in persisting infections. Similarly, in a recent study using a sophisticated nested PCR technique, the target bacterium E. faecalis was found in 41 of 50 (82%) untreated root canals and in 38 of 50 (76%) treatment failure associated root canals (31). As in other related works (32– 35), PCR methodology seems to be exclusively directed to find only E. faecalis, ignoring the rest of the flora present that may be as important as E. faecalis in provoking the treatment failures.

On the other hand, recent investigations have confirmed the polymicrobial nature of root canal infections (36, 57). In a study with monkeys (36), different combinations of bacteria were experimentally inoculated in root canals and periapical lesions were induced. The teeth were treated endodontically and followed-up radiographically and histologically for 2 to 2.5 years. In the root canals with bacteria present when the root filling was removed, 30 of the 31 canals had persisting periapical lesions. Importantly, more of these non-healed lesions were associated with various combinations of bacterial strains, that is, mixed infections, than single strains. Previously, the same research group (38) also found that when an “eight-strain collection” of species, derived from one infected root canal, was re-inoculated in equal proportions into other monkey teeth, species such as Bacteroides oralis (now Prevotella oralis) dominated in mixed

infections and showed a more potent capacity for tissue destruction. Furthermore, B. oralis could not be reisolated from inoculated root canals after the experimental period when inoculated as a pure culture. In another study using the tissue cage model implanted subcutaneously in the backs of rabbits, the same col- lection of eight bacterial strains from monkey root canals were inoculated in different combinations and individual species. The combination of B. oralis, Fusobacterium necrophorum, Peptostreptococcus anaerobius, and Streptococcus milleri was the most predominant and induced higher titers of circulating antibodies than that obtained with individual inoculations, such as E. faecalis (39).

Even if we accept the polymicrobial nature of root canal infections, one of the major problems in understanding endodontic infections is that we still extrapolate between individual organisms growing in liquid (planktonic) cultures and the in vivo situation. A significant literature now exists demonstrating that the physiology of a bacterium in planktonic culture is profoundly different from that of the same organism growing on a surface in a biofilm [see review by Costerton et al. (40)]. For instance, planktonic bacteria are more sensitive to antimicrobial agents because of their ease of diffusion within the bulk fluid, whereas biofilm bacteria are notably resistant to these agents (41–45). In this context, the study of biofilms in root canal infections has included biofilms formed by mixed cultures of anaerobic bacteria in extracted teeth (46, 47) or by pure cultures of E. faecalis (48, 49). Biofilms of five root canal isolates have also been used to test the antimicrobial efficacy of endodontic irrigants, such as sodium hypochlorite (NaOCl) (2.25%), 0.2% chlorhexidine, 10% povidone iodine, and 5 ppm colloidal silver, with NaOCl shown to be the most effective agent of this group (50). In addition, Chavez et al. tested the alkaline tolerance of species isolated from chronically infected root canals and found that E. faecalis and other Gram-positive organisms, such as Lactobacillus paracasei, Olsenella uli, or Streptococcus gordonii, shared similarly high alkaline-tolerant capabilities when

growing in planktonic conditions. S. anginosus, S. oralis, and F. nucleatum, on the other hand, were greatly affected by the alkaline stress (see Fig. 1) (51). Of importance, however, was the observation that this difference in alkaline tolerance was not apparent when the strains were tested in biofilms because all seven strains showed a similar high tolerance to alkaline pH (Fig. 1). These findings not only show the capacity of root canal bacteria other than E. faecalis to adapt to alkaline stress, but also provide further evidence that bacteria in surface-adhered biofilm consortia are more resistant to environmental stress than when grown in liquid culture.

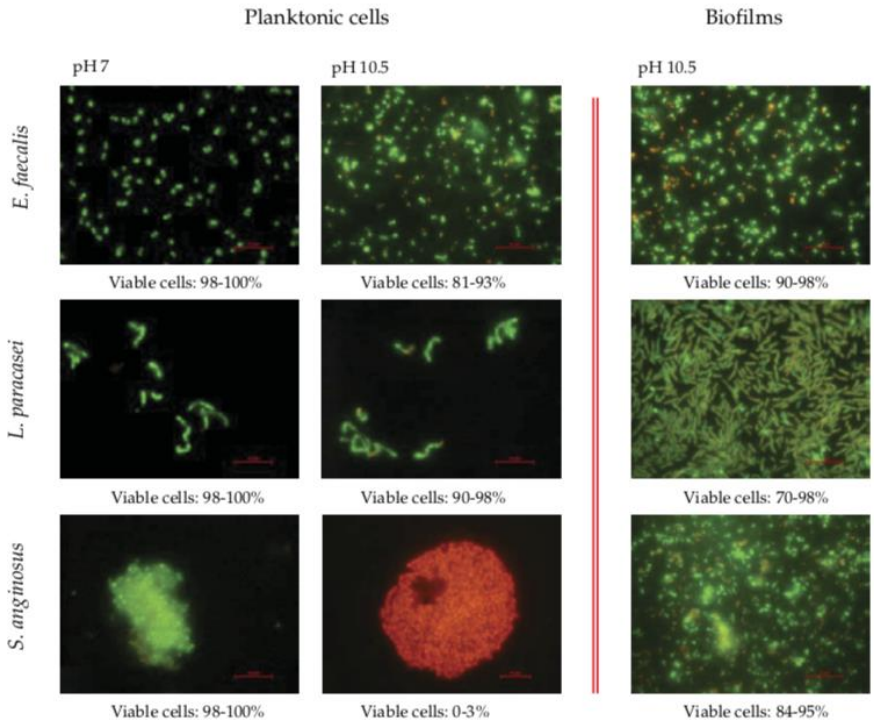

Figure 1. Fluorescence micrographs using Live/Death fluorescence staining for bacterial viability. Cells stained fluorescent green represent viable cells, whereas cells stained fluorescent red are nonviable or damaged. In the first column, images show planktonic cells of three root canal strains at neutral media (pH 7). The middle column shows planktonic cells after exposure to pH 10.5 for 4 hours, and the right column shows biofilm cells exposed to alkaline challenge (pH 10.5) for 4 hours. Bars, 2 m. Images are published with permission of Blackwell Publishing. International Endodontic Journal, Chávez de Paz et al. (65)

As the host defense loses its access to the necrotic pulp space, opportunistic microorganisms selected by harsh ecological conditions and the low-oxygen environment aggregate in the root canal system (52). These microbial communities may survive on organic pulp tissue remnants and exudate from the periodontium (53, 54). Consequently, clusters of microorganisms in necrotic teeth and teeth with failed root canal treatments are typically found in the apical root canal area, where they have access to tissue fluid (52). In long-standing infections, root canal bacteria can invade the adjacent dentin via open dentinal tubules (55, 56).

Primary root canal infections are polymicrobial, typically dominated by obligately anaerobic bacteria (53). The most frequently isolated microorganisms before root canal treatment include Gram-negative anaerobic rods, Gram-positive anaerobic cocci, Gram-positive anaerobic and facultative rods, Lactobacillus species and Gram-positive facultative Streptococcus species (53). The obligate anaerobes are rather easily eradicated during root canal treatment. On the other hand, facultative bacteria such as non-mutans Streptococci, Enterococci, and Lactobacilli, once established, are more likely to survive chemomechanical instrumentation and root canal medication (57). Enterococcus *faecalis* has gained attention in the endodontic literature, as it can frequently be isolated from root canals in cases of failed root canal treatments (58, 59). In addition, yeasts may also be found in root canals associated with therapy-resistant apical periodontitis (60).

It is likely that all the microorganisms able to colonize the necrotic root canal system cause periapical inflammatory lesions. Enterococci can survive in monoculture but cause only minor lesions (38). Certain Gram-negative taxa appear to be more virulent (53). The outer membrane of Gram-negative bacteria contains endotoxin, which is present in all necrotic teeth with periapical lesions (61) and is able to trigger an inflammatory response even in the absence of viable bacteria (62). Furthermore, the levels of endotoxin in necrotic root

canals are positively correlated to clinical symptoms such as spontaneous pain and tenderness to percussion (63). Virulent Gram-negative anaerobic rods depend on the presence of other bacteria in their environment to survive and establish their full pathogenic potential (38). Such aggregations of microorganisms in an extracellular polysaccharide matrix associated with a surface (in our case the inner root canal wall) are called biofilms (64). There is convincing evidence that microorganisms organized in this manner are far less susceptible to antimicrobial agents than their planktonic counterparts, which have traditionally been used to test the antimicrobial efficacy of substances in vitro (65, 66). If a bacterially inoculated broth is confronted with an antimicrobial fluid, the efficacy of that agent can appear to be very convincing, similar as with agar-diffusion tests. However, in the root canal system biofilms and infected dentinal tubules make disinfection much more difficult and thus study models such as standardized infected bovine dentin blocks (67) or in vivo models appear to be more valid than the above-mentioned study designs. Furthermore, it has been shown that organic and inorganic dentin components, which are suspended in the irrigants during chemo-mechanical instrumentation, inhibit most antimicrobial agents (68, 69).

In conclusion, the biofilm concept and the specific conditions in the pulpless root canal cannot be overestimated when considering the actions of different irrigating solutions.

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1.2 ROOT CANAL INSTRUMENTATION

The objectives of mechanical preparation are two-fold:

1. To facilitate irrigation Conventional radiography does not enlighten the clinician about the true complexity of the root canal system. Lateral canals, fins, anastomoses, and ramifications are invariably present, with some canals being joined by narrow isthmus. The main canal is rarely round, but often oval, ribbon- like or even 'C'-shaped, depending upon the tooth. One seminal study has demonstrated up to 53% of the canal will remain unreached by instrumentation following preparation (1). Therefore, mechanical preparation facilitates penetration of irrigants into these complex anatomical spaces. Although some dentine-containing micro-organisms will be removed during mechanical preparation, research suggests that a considerable amount of the canal will not be contacted by a file, therefore irrigants play a crucial role in destroying micro-organisms, neutralizing endotoxin, and removing organic tissue components (2).

2. To facilitate obturation as cleaning and shaping does not remove all micro-organisms from the canal, obturation aims to entomb any residual pathogens and limit recolonization by preventing the passage of nutrients from both coronal and apical aspects. Mechanical preparation facilitates obturation. Schilder's principles of canal preparation still hold true today (3). The idea of creating a continuously tapering preparation, free from mechanical errors, allows the best chance of a well-condensed obturation, with the absence of voids.

1.2.1 The crown down approach

Most microorganisms are in the coronal portion of the canal and pulp chamber (4). Thus, whatever instruments are used, a crown down approach and only initial scouting of the canal prior to working length determination is sensible. This technique involves shaping the canal from the coronal aspect first and progressively working more apically

with smaller diameter instruments (5) (Figure 2).

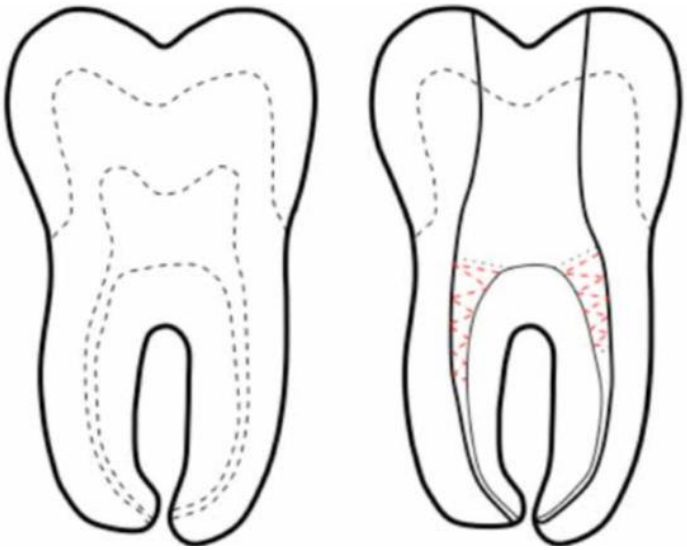

Figure 2. The crown down approach: the coronal third of the canal system is enlarged using GG or orifice-shaping files. The enlargement is directed away from the furcation and has the simultaneous benefit of removing dentine overhanging the orifices to allow optimal straight-line access.

Such an approach:

- Minimizes the transportation of pathogens further into the canal system.
- Allows a greater amount of irrigant to be held in the canal, facilitating debris removal and disinfection.
- Removes coronal curvatures and facilitates straight-line access.
- Improves accuracy of working length determination as reduction of curvature after working length determination may alter the working length and result in a tendency to transport the canal and over-enlarge the apical foramen.
- Reduces file binding in the coronal portion of the canal, facilitating working length assessment and further reducing the risk of instrument separation through torsional failure.

Traditionally, Gates Glidden (GG) instruments would be used for the crown down procedure, but many rotary filing systems now have orifice shapers to begin the preparation. If clinicians elect to use GGs it is wise to remember a Size 6 GG has an apical diameter of 1.5 mm (ISO 150), with sizes stepping down in 0.2 mm increments to a Size 1 GG at 0.50 mm (ISO 50). As such, even the smallest of GGs can be very destructive if used carelessly. Avoid using sizes above GG 3 (0.90 mm: ISO 90). Whatever instruments are used, caution must be taken regarding the furcation region, the instruments being used away from the furcation (anti-curvature filing) (6). Despite the advantages, it is easier to create blockages and ledges with an aggressive or careless crown down approach, thus highlighting the importance of recapitulation.

1.2.2 Working length determination

The apical extent of preparation should be kept within the canal system: over extension can reduce success up to 62% and, for every mm short of the apex, underextension reduces success by 12% (7). Methods used to estimate the maximum working length for instrumentation include apical gauging by tactile sensation, instrumentation without local anaesthetic, using pre-operative radiographs alone, the paper point technique, working length radiographs (WLRs) with files *in situ* and, most recently, the use of electronic apex locators (EALs). Historically, the most widely accepted method is by placing a file to the estimated length, then taking a confirmatory radiograph, but the radiographic apex rarely corresponds with the anatomical apex (8) It follows that WLRs can only give an estimation of the correct termination of preparation.

Modern impedance-based multifrequency EALs are reliable and accurate >90% of the time (9) These devices are only accurate at a ZERO reading. Any reading given other than ZERO should not be used as a marker of apical extent. The ZERO reading is reached when

the file contacts the periodontal ligament. Thus, by definition this is over extended and, to calculate the working length, one must subtract 0.5 mm from the ZERO reading length (10). For more information readers are referred to other papers on the subject of EALs (11,12).
The 2013 Faculty of General Dental Practitioners Selection Criteria for Dental Radiography states '*Unless there is confidence about working length(s) derived from an electronic apex locator, at least one good-quality radiograph is necessary to confirm working length(s)*' (13). From this one could extrapolate that WLRs are no longer always necessary. We recommend that a combination of techniques is used.

1.2.3 Size of apical preparation

There is equivocal evidence regarding the effect of the size of apical preparation on the success of endodontic treatment (14,15) Smaller apical preparation has the advantage of minimizing the risk of transportation and extrusion of debris and irrigant. Conversely, a more aggressive apical preparation will remove more infected dentine and allow greater access to irrigants but may increase the risk of perforation and extrusion of debris and irrigants. Traditional teaching advocated using a master apical file which was three sizes larger than the first file to bind (16) Subsequent work has shown this method to be inaccurate (17). In addition, most apical foramina are not round but ovoid in shape and it is questionable whether infected dentine needs to be removed as appropriate irrigation penetrates dentine and kills micro-organisms (18). A modern approach to apical enlargement focuses on irrigation. Irrigant must reach the apical 1 mm of the canal (19). Evidence suggests that irrigants do not flow greater than 1−2 mm past the syringe tip. Ideally, the irrigating syringe tip must be

placed within 1−2 mm of the apex (20). A conventional 30 gauge needle corresponds to the tip of an ISO 30 file, therefore an apical preparation smaller than this may result in the inability to place the needle tip within the apical 2 mm and thus there may be inadequate irrigation in this area. We suggest that an apical preparation of 0.25−0.30 mm (ISO 25−30) should be considered a good target. In addition, it has been demonstrated that larger taper preparations enhance cleaning and irrigation and subsequently reduce bacterial load (21). One study has shown only modest increases in irrigation with taper increases beyond 0.04 (22). The clinician must therefore be aware that increasing taper carelessly may also increase the risk of excessive tooth structure removal and perforation without added benefit. If canals are sclerosed or very curved such large preparation may not be possible.

1.2.4 Preparation techniques

New endodontic instrumentation systems are being continually introduced on to the market, allowing clinicians to complete endodontic treatment with simpler protocols, faster. Accordingly, there has been a paradigm shift towards nickel titanium rotary file systems. Nonetheless the clinician must understand the importance of hand filing: the clinician that cannot hand file is handicapped in the ‘art of endodontics’.

1.2.5 Hand file instrumentation

Hand files afford the clinician greater tactile feedback than rotary instruments and are often invaluable in determining the direction and magnitude of curvatures and canal configurations. There are two main types of files: Hedstrom and K files. The former are machined stainless steel cylinders that cut aggressively. The latter are twisted stainless steel that are more flexible and less aggressive. The cross-

section varies depending on the type of file. All have 16 mm fluted portions and follow ISO dimensions. New instruments are available in nickel titanium. These instruments are flexible and potentially safer but cannot be pre- curved and negate some of the benefit of hand filing in the early stages, especially in curved canals.

Shaping the canal with hand files can be undertaken in numerous ways, depending upon the canal anatomy . Techniques for total canal preparation with hand files includes 'step-back', 'crown- down', 'double flare' and 'anticurvature filing' (5,6,23,24). Techniques for manipulation of the files during preparation include circumferential filing, 'balanced force' (25), watch-winding and push-pull. Thus the former describe the strategy and the latter describes the method of achieving that. 'Step-back' and 'double flare' techniques both involve determining the working length and choosing a master apical file size, then using progressively larger files at shorter lengths in order to create a continuous taper. Stainless steel hand files are all standard 2% ISO taper. The operator can choose the degree of taper created by adjusting the lengths to which progressively larger files are inserted. Traditional step back, using increments of 1 mm creates a canal with a 5% taper. If the clinician wishes to develop a larger taper, then reducing the increments to 0.5 mm will result in a canal with a 10% taper. One common pitfall with both these techniques is under preparation of the middle third of the canal. This poses problems when obturating using cold lateral compaction techniques, as accessory points cannot penetrate past the coronal third, resulting in an obturation which resembles an 'inverted wine bottle'.

The 'balanced force' technique involves turning the file clockwise up to 90° followed by an anti-clockwise movement of 180° or more whilst maintaining apical pressure (25). The first movement engages the dentine, whilst the second movement releases and cuts the canal wall. This permits predictable, centred dentine removal. Though 'balanced force' may be used in all canals, it is an especially effective and safe technique for hand filing curved canals.

Circumferential and push-pull filing techniques are more suitable for straight, wide canals, C-shaped or ovoid canals: the walls of the canal are reamed with an oscillating apico-coronal movement. As a rule, the use of stainless steel endodontic instruments should be avoided in rotary hand-pieces as they can be aggressive and are prone to breakage.

Stainless steel files may be pre-curved to the estimated shape of the canal, preferably with a designated instrument to avoid contamination . It is useful to indicate the direction of the curve by marking it with the pointer on the rubber stop. After using each successive file, always irrigate and recapitulate with a fine file, such as #10, to disrupt and to agitate the plug of 'dentine mud' which builds up apically which can result in loss of working length.

1.2.6 Patency filing

Patency filing is the process of placing an ISO 10 file (or smaller) 0.5 mm passively beyond the apex (26). It is imperative that the file is not excessively rotated, as this can enlarge the apical foramen. This removes dentine plugs that can be compacted in the apical region. These can harbour bacteria and may result in deviation of the instrument tip if not cleared. Ensuring patency of canals improves the success of RCT7.

1.2.7 The era of nickel titanium

The most notable development in endodontics in the last 25 years is the introduction of nickel titanium (NiTi) instruments (27) This alloy, composed of 55% nickel and 45% titanium has several properties which are desirable for endodontics; most notably, NiTi has super elasticity and shape memory. This helps to keep the file centred in the canal and reduces the risk of procedural errors. Although NiTi instruments are commonly associated with rotary techniques, many

manufacturers also produce hand file versions of their rotary systems, which are designed to be used in the same sequence. The super elasticity of nickel titanium does, however, prevent these files being pre-curved. Recent advances in material technology now afford greater flexibility and cyclic fatigue resistance (28) These include *M-wire* (Dentsply, Tulsa) and *HyFlex CM* or *Controlled Memory* (Coltene/Whaledent, Germany). *M-wire* is now used in the production of single file systems (see below). *HyFlex CM* instruments can also be pre-bent, reducing the risk of ledging, transportation or perforation. This may potentially revolutionize nickel- titanium technology.

1.2.8 Rotary file systems

Since the introduction of nickel titanium it has been possible to prepare root canals using a motor safely and predictably. Rotary instrumentation increases cutting efficiency. Although speed reducing motor hand-pieces can be coupled to existing units, the use of dedicated electric endodontic motors is recommended. The torque and speed can be adjusted to match the instrument manufacturers' specifications precisely and many have auto reverse to prevent files binding in the canal and exceeding the torque limit. Rotary files usually create preparations of greater taper than the conventional ISO 2%, with some systems exhibiting variable taper throughout the length of the file.

Although most practitioners will be familiar with the manufacturers' protocol for such instruments, Table 1 offers a list of guidelines relevant to all using rotary instrumentation (29).

■ Create straight line access to the coronal or middle third of the root before using a hand or rotary instrument
■ Create a glide path up to a #20 hand file to the apex before using hand or rotary instruments in that part of the canal
■ Fine files frequently: after 3–4 pecks of a rotary instrument, remove, irrigate and recapitulate with fine files
■ Thoroughly clean the flutes of the instrument after removal from the canal
■ Maintain patency throughout by taking a small (ISO 8 or 10) file 0.5 mm beyond the working length
■ Never force a rotary instrument. If resistance is met; stop, increase the amount of coronal flaring, irrigate and recapitulate
■ If challenging anatomy is present, always prepare the canal with hand files before introducing rotary instruments
■ Don't try to bypass ledges with rotary instruments; always use hand files for this
■ Prepare sufficient coronal flaring to ensure that the minimum amount of the file is contacting the canal walls
■ Always introduce the file into the canal whilst it is rotating. Do not stop and start the motor once the file is in the canal
■ Make sure that you have an accurate working length before using rotary files in the apical area. Aggressive enlargement and transportation can occur if a rotary file goes beyond the apex
■ Any time saved in preparation should be used in irrigation

Table 1. Tips for using rotary NiTi file systems modified from the AAEs Guidelines.

Most manufacturers would recommend the use of a 'glide path' to ensure safe and efficient passage of the instruments to full working length. By taking an ISO 20 hand file to the length to which a NiTi instrument is to go will significantly reduce the risk of instrument fracture, as covered below. There are ranges of NiTi instruments that are advocated for developing a glide path (eg *Pathfile* (Dentsply, Tulsa, USA). The manufacturers indicate these for use in sclerosed or difficult to negotiate canals. These should be used at slow speeds and with caution. It remains sensible to create a glide path with hand instruments first. The finer details of file design and shape will not be covered in this paper but the clinician should be aware that many of the properties of an instrument are not simply governed by the material but the shape of the instrument. It is important to know the cutting efficiency, the taper size, and the instrument diameters at the tip. Although rotary NiTi file systems can be advantageous for preserving the original canal anatomy, they have limitations: When straight files are placed into curved roots the instrument can straighten the canal, resulting in a 'zip' apically where the apex is expanded. This is virtually impossible to fill. Rotary instruments should not be left rotating for more than 3–4 pecks of the apex to prevent such zipping and the ensuing difficulties this presents for obturation. Rotary preparations are circular, thus they are less useful in ribbon and 'C'-shaped canals, which are better prepared with hand files using

circumferential techniques. Rotary files have a propensity to separate by two mechanisms (30) First, torsional failure can occur by the file continuing to rotate whilst one part of it is bound against the canal. Secondly, continuous rotation of the file in a curved canal can result in cyclical failure. The move to single use instruments reduces the risk of instrument separation but this will never mitigate the risks of poor technique. Always inspect the tips of instruments during use: if the threads are unwinding there is a risk of separation, so discard them. Nonetheless, NiTi rotary instrumentation is safe and effective if care is taken and manufacturer's instructions are followed (31).

1.2.9 Reciprocating systems

Reciprocation involves the file rotating in both anti-clockwise and clockwise directions: essentially a form of mechanized 'balanced force'. The anti- clockwise movement engages dentine following which the clockwise turn releases the file from the canal before re-engaging the canal wall, shearing dentine and creating the preparation. The reciprocating motion and single file system has several important benefits: Decreased risk of cyclical failure as the files are rotating at a lower RPM; Decreased risk of torsional failure as the filing motion repeatedly disengages the dentine, thus preventing binding and instrument fracture; More cost-effective endodontic treatment as the current reciprocating systems are 'single file'. A simplified protocol with only three choices of instrument for small, regular or large canals. Currently, there are two systems on the market, *Wave One* (Dentsply- Maillefer, Ballaigues, Switzerland) and *Reciproc* (VDW, Munich, Germany). *Wave One* utilizes an 170°:50° anti- clockwise: clockwise movement and *Reciproc* 150°:30°. This means that it will take three reciprocating movements for both file systems to rotate 360°. Although marketed as a single file system, the ecommended protocol for *Wave One* still involves the initial use of hand files (32). The manufacturers of *Reciproc* advocate that

production of a glide path with hand files is not required in most cases (33). It remains good practice to establish a glide path with 0.20 ISO files before any NiTi instrument is used to working length. These instruments surpass conventional rotary instruments in resisting cyclical and torsion fatigue and, although similar in concept, *Wave One* has greater resistance to torsional fatigue than *Reciproc* and *Reciproc* has greater resistance to cyclical fatigue than *Wave One* (34). This means that *Reciproc* is more suited to curved canals and *Wave One* to narrow or sclerosed canals.

1.2.10 Which system is best?

The method of instrumentation used (hand or rotary) does not appear to influence success rates (7), although one study found better success rates with rotary instruments amongst general practitioners (39). Although manufacturers are becoming more aware of the importance of robust supporting evidence, clinicians must not be duped by the marketing and should research the systems independently, if possible. We recommend practitioners remain open-minded about using differing systems using extracted teeth to trial new filing systems. Finally, always remember the mantra ‘files shape and irrigants clean’: no system of instrumentation renders the canal bacteria free (40,41) Irrigation is the key to success in endodontics and will be discussed in the next chapter.

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IRRIGATION

During endodontic treatment mechanical debridement alone will not rid the root canals of bacteria (1) regardless of whether this is done by hand files or rotary instruments (2). First, instruments do not access the complex shape of the root canal system (3-6). Secondly, within these inaccessible regions complex biofilms can develop that are not easily disrupted. Thirdly, instrumentation creates a smear layer that further prevents decontamination of the canal surface dentine and prevents a good adaptation of the obturation material to the canal wall. A sound irrigation regimen can help to deliver antimicrobials to these inaccessible areas of the root canal system, penetrate and remove biofilm and smear layer and even penetrate the dentine.

2.1 Type of irrigant

A recent Cochrane Systematic Review showed no difference between different endodontic irrigants (7). However, these results should be interpreted with caution. A 'no difference' result reflects the paucity of well-conducted clinical studies rather than taking as fact that no difference exists. The irrigant has several primary goals: dissolution of organic tissue and pulpal remnants, be they vital or necrotic, dissolution of select inorganic components, killing of micro-organisms and neutralization of endotoxin.

Many different irrigants and combinations of irrigants have been used in RCT to achieve these goals. These include:

- Sodium hypochlorite;
- Chlorhexidine;
- Sterilox;
- EDTA;
- Iodine potassium iodide;
    - Hydrogen peroxide;
    - Local anaesthetic, saline and/or water;

- Mixtures of irrigants (QMIX®).

See Table 1 for a summary of their differing properties (8). When used alone, very few irrigants offer a complete spectrum of ideal properties.

	TYPE	ACTION ON FLORA	TISSUE DISSOLUTION	ENDOTOXIN DEACTIVATION	INORGANIC SMEAR LAYER	SUBSTANTIVITY	TOXIC?	ALLERGENIC?	COST
SODIUM HYPOCHLORITE >1%	HALOGEN IONS	✓✓	✓✓✓	✓	X	X	X	-VE	£
CHLORHEXIDINE 0.2%	BISGUANIDE	✓✓	X	✓	X	✓	?	+VE	££
HYPOCHLOROUS ACID		✓✓✓	X	?	X	X	✓	?	££
EDTA	CHELATING AGENT	✓	X	X	✓✓✓	X	✓	-VE	££
IODINE POTASSIUM IODIDE	HALOGEN IONS	✓✓	X	X	X	X	✓	+VE	££
HYDROGEN PEROXIDE	PEROXIDE	✓	X	X	X	X	X	-VE	£
SALINE/WATER/ LOCAL ANAESTHETIC	PLACEBO!	X	X	X	X	X	✓	-VE	£

Table 1. Commonly used irrigants and their key properties. Modified from Zehnder 2006 (8).

Sodium hypochlorite

Sodium hypochlorite (NaOCl) was first described as an endodontic irrigant in 1919 (9). It possesses many of the attributes of an ideal antimicrobial agent; it is fast acting, has a broad spectrum of action and is relatively inexpensive (10). Its activity stems from several key aspects. Hydroxyl ions damage both bacterial lipid membranes and DNA and the high pH created denatures proteins and impairs ideal cell conditions. Chloride ions break peptide bonds dissolving protein and releasing further chloramines that are antibacterial. It remains the gold standard of endodontic irrigants and, although bacteria can still be cultured following irrigation with hypochlorite, it is nevertheless more effective than saline (11,12). It should be stored in a cool, dark, air-tight and non-reactive bottle.

Chlorhexidine

Chlorhexidine (CHX) has a broad-spectrum activity against both Gram positive and Gram negative bacteria and is also antifungal. Its antimicrobial activity results from the disruption of bacterial cell walls. Furthermore, it has substantivity; it bonds to dentinal walls, maintaining its antibacterial properties for up to 12 weeks. Chlorhexidine has been used as a substitute for hypochlorite (especially by non-rubber dam users). However, it remains inferior as it does not possess the capacity to dissolve organic matter, and its effect on microbial biofilms is less than that of hypochlorite (13). Practitioners must also be aware that CHX at 0.2% (found in proprietary mouthwashes) concentrations is only bacteriostatic; to have a bactericidal effect concentration of 2% must be used. There is also evidence that it can have a negative effect on healing, resulting in an increased odd of failure (14). In addition, there is a growing concern with CHX and sensitization that may result in anaphylaxis (15).

Sterilox

Sterilox is a solution that is a safe, non-toxic broad-spectrum biocide. The main active ingredient in Sterilox is hypochlorous acid at a concentration of 200 ppm of available free chlorine. Hypochlorous acid has been reported to be many times more effective than hypochlorite (bleach) as a biocide. Sterilox solution has approximately 85−98% hypochlorous acid. As a root canal irrigant it has little or no tissue dissolving properties, however, it is non-toxic and safe to use where there may be an open apex and is preferable as an antibacterial agent compared with NaOCl (16).

EDTA

Ethylenediamine tetra acetic acid (EDTA) (17%) is a chelating agent which removes inorganic debris. It has been found to be beneficial in removing the smear layer and preparing the canal for obturation.11 EDTA should be used as a final rinse, with no hypochlorite thereafter (17). In retreatment cases it facilitates the removal of the smear layer and GP residue. It should be used as an adjunct to sodium hypochlorite, not a replacement. It has low toxicity. An alternative is 10−50% citric acid. It too removes the smear layer and is safe.

Iodine-potassium-iodide

Iodine-potassium-iodide (IKI) has been used as an endodontic irrigant. It has excellent antimicrobial activity and low toxicity (18). It is available in 2% iodine or 4% potassium iodide. Like sodium hypochlorite, it has the ability to penetrate dentinal tubules to a greater extent than chlorhexidine (19). It can stain dentine and may cause allergic reaction, so it is advisable to take an allergy history of the patient before using.

Hydrogen peroxide

Hydrogen peroxide (H2O2) has a long history of use in endodontics. It has been used in concentrations between 3 and 30% (20). It is active against bacteria, viruses and yeasts (21). Despite this, the available evidence does not support the use of H2O2 over other irrigants and its use is no longer recommended (22).

Mixtures of irrigants

MTAD and QMIX® have been developed more recently. Both contain surfactants that may lower the surface tension of the irrigant and promote penetration within dentine. MTAD consists of doxycycline, citric acid and detergent.

A recent review of this irrigant outlines its properties and explains that the solution shows promise as an endodontic irrigant in terms of excellent smear layer removal, less concomitant negative effects on dentine, and good biocompatibility (23). However, there is limited clinical evidence to support the use of MTAD (24). If used, it should be regarded as an adjunct to NaOCl, not a replacement (25). QMIX® is a mixture of chlorhexidine, EDTA and a surfactant. As previously highlighted, this solution will not dissolve organic debris and is thus of limited application alone.

Local anesthetic, saline and/or water

Practitioners using water, saline or local anesthetic are profoundly misguided in their understanding of endodontics and place themselves at risk of litigation should treatment fail: these irrigants have no antimicrobial properties. Of the many irrigants on the market, sodium hypochlorite remains the gold standard. It is readily available from dental suppliers and is CE marked. Use of 'thin' household bleach was advocated in the past (26) letting it down 1 part bleach to 2 parts sterile/distilled water to give a 1% solution. In this increasingly litigious world, however, it would be wise to use only CE marked 'bleach for dental use'. Although other irrigants exist, they should be regarded as adjuncts, not alternatives.

Irrigant mixing

As a rule, the mixing of irrigants should be avoided. Do not mix chlorhexidine and hypochlorite. When mixed they form a precipitate, parachloroaniline which is thought to be carcinogenic, it may stain the

tooth and will block the canal and tubules from further irrigant effect (25). Repeated cycling of hypochlorite and EDTA should be avoided as this will erode dentine and compromise tooth structure (11). EDTA should be used as a final irrigant once canal preparation has been concluded (25).

Factor influencing endodontic irrigants

All concentrations of hypochlorite are superior to saline, though there is little difference in efficacy between concentrations (27). Although 0.5% concentration of NaOCl has been shown to be no different from 5% in terms of bactericidal actions, a concentration of at least 1% (neat Milton is 2%) is required for tissue dissolution (28). There have been concerns about the safety of concentrations greater than 1%, especially when patency filing. The lower the concentration, the less the risk of a hypochlorite accident. However, no direct link between concentration of hypochlorite and subsequent tissue damage has been made, as a hypochlorite accident is regarded as a scenario with multifactorial aetiology (29). Chlorhexidine is only bacteriostatic at 0.2% (proprietary mouthwash concentration), but at 2% is bactericidal. Practitioners should be aware of this distinction when considering hypochlorite alternative.

Heating 1% hypochlorite has been shown to improve its properties. A 2.6% solution of NaOCl at 37oC is as effective as 5.2% at 22°C at both tissue dissolution and bacterial killing (30). Therefore, less cytotoxic concentrations can effectively be used and increasingly practitioners have been heating syringes of hypochlorite in a waterbath prior to use to maximize its effect.

The longer the irrigant is in contact with root surfaces, the greater the likelihood of successfully killing microbes and reducing the bacterial load. In wider canals, where there is less emphasis on mechanical shaping, longer periods of contact with irrigants are necessary (11,31). The chlorine component of hypochlorite rapidly

depletes and may no longer be active after 2 minutes (32). Constant irrigant exchange throughout treatment is thus essential.

It has been shown that exceeding a rate above 4 ml/min does not improve apical clearance but does increase the risk of extrusion; (33) therefore 1 ml increments over 15 seconds give maximum exchange and minimum risk. Further evidence suggests that irrigant does not move further than 1−2 mm beyond the needle tip in the canal irrespective of the pressure applied. Beyond this there is a 'dead zone' and irrigant exchange does not occur, thus consideration must also be given to mode of delivery (34) .

Mode of delivery

An irrigant may not always access anatomical irregularities, remove debris, and eradicate those pathogens embedded in biofilm. Thus, there is growing evidence that irrigant alone may not be adequate and consideration must also be given to exchange and agitation of an irrigant to facilitate decontamination (35). Circulation and removal of the irrigant and debris are essential components of the cleaning protocol. There are various techniques for ensuring optimal delivery and exchange.Direct injection (positive pressure) is the most common technique for introducing irrigant into canals. A syringe is introduced, and pressure applied to deliver irrigant into the canal. The clinician must aim to deliver irrigant to within 1 mm of the apex (36). Access to the apex is dependent on the size and taper of the canal in question. A 27G needle placed 3 mm from the apex of a canal prepared to 0.3 mm (ISO 30) is sufficient (37). An irrigant must not be forced into the root canal system. Forefinger pressure as opposed to thumb pressure is advisable. Although increasing the diameter of the syringe will improve irrigation, this must be balanced with the desire to deliver the

syringe tip to within 3 mm of the apex. Do not allow the needle tip to lock in the canal.

Positive pressure irrigation has been associated with two drawbacks:

1. Risk of extrusion; and
2. Inability to irrigate the apical region.

The former can be reduced by a safe irrigating technique, described below. The latter is thought to be due to the formation of bubbles of air within the canal, blocking irrigant penetration; a phenomenon known as vapour lock (38). This problem can be minimized by using a patency filing technique or negative pressure irrigation (39). In addition, in narrow curved canals, introduction of a syringe apically may be impossible. Many manufacturers sell flexible tips that can negotiate curved canals more easily.

The EndoVac (SybronEndo, Orange CA) involves the use of anirrigant delivery cannula combined with a microsuction system. The aspirating cannula draws irrigant into the canal by creating a negative pressure. Thus there is continual irrigant exchange with reduced risk of extrusion. Developed to counteract the difficulties of positive pressure irrigation, this has been shown to improve apical irrigation safely (40). This is not without limitations:

1. Debris left in situ (41)
2. Larger apical preparations up to ISO 40 are required to permit introduction of both cannulae (and this may not be possible in curved canals) (42,43)
3. Cannulae may block with debris (44)
4. Creating a preparation coronally that allows effective adaptation of the system to the canal can be challenging.

2.4 Activation of irrigant

Ultrasonic: The application of ultrasonic energy (20−26 kHz) to files within the canal generates acoustic streaming of the irrigant (45). This increases the turbulence of flow, improving distribution of irrigant,

penetration into isthmuses and tissue dissolution. This must be done upon completion of shaping and an ISO 20 or 15 file introduced passively (contact with the canal walls should be avoided as it can remove dentine and create ledges).

A protocol of passive ultrasonic irrigation (PUI) has been suggested of 3 x 20 second cycles per canal and the file may be used in an in-out motion (46). Dedicated systems are available (MiniEndo, Spartan EIE Inc, San Diego CA) but the clinician may simply touch the file with an ultrasonic tip. Though this works well in large straight canals, there is mixed evidence regarding the benefits (47).

Sonic: The application of sonic energy (1−6 kHz) is thought to have a similar effect to ultrasonic irrigation. The EndoActivator (Dentsply, Tulsa OK) uses disposable polymeric tips with length markings in a battery-powered hand-piece which is theoretically safer to use. Although both sonic and ultrasonic agitation improve cleaning over conventional techniques, they still leave debris within the canal (48).

The multisonic ultracleaning system (EMS, Dallas, Texas) uses multiple sonic waves to facilitate irrigation. It will be marketed as 'Gentle Wave' (Sonendo, California USA). It operates using a hand-piece but no component of this is placed within the canal system. The instrument is placed over the pulp chamber, sealing the tooth from the oral cavity and is activated from a computer console. From a spray of irrigant is delivered at 45 ml/ min at 40 °C. Early in vitro results are interesting, with the system showing higher rates of tissue dissolution with differing concentrations of hypochlorite and water (49).

Following completion of shaping, the canal is filled with irrigant and the GP master-cone inserted. It is then 'pumped' up and down in rapid 3 mm motions. This can overcome 'vapour lock' and facilitate irrigant exchange close to the FWL, while at the same time disinfecting the GP cone prior to cementation.

Conclusion

Thorough irrigation of the canal system is essential in endodontics. For those clinicians not using sodium hypochlorite as the principal irrigant it must be acknowledged that, as yet there is no other irrigant that offers all the benefits of hypochlorite and is as cost-effective. It may be the fear of extrusion that deters practitioners, but, with a careful technique, the risk of this complication can be reduced significantly.

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The role of Smear layer

Whenever dentine is cut using hand or rotary instruments, the mineralized tissues are not shredded or cleaved but shattered to produce considerable quantities of debris. Much of this, made up of very small particles of mineralized collagen matrix, is spread over the surface to form what is called the smear layer. Identification of the smear layer was made possible using the electron microprobe with scanning electron microscope (SEM) attachment, and first reported by Eick et al. (1970). These workers showed that the smear layer was made of particles ranging in size from less than 0.5–15 lm. Scanning electron microscope studies of cavity preparations by Bra¨nnstro¨m & Johnson (1974) demonstrated a thin layer of grinding debris. They estimated it to be 2–5 lm thick, extending a few micrometres into the dentinal tubules.

The smear layer in a cavity and in the root canal may not be directly comparable. Not only are the tools for dentine preparation different in coronal cavities, but in the root canal the dentinal tubule numbers show greater variation and there are likely to be more soft tissue remnants present. The first researchers to describe the smear layer on the surface of instrumented root canals were McComb & Smith (1975). They suggested that the smear layer consisted not only of dentine as in the coronal smear layer, but also the remnants of odontoblastic processes, pulp tissue and bacteria. Lester & Boyde (1977) described the smear layer as ‘organic matter trapped within translocated inorganic dentine’. As it was not removed by sodium hypochlorite irrigation, they concluded that it was primarily composed of inorganic dentine. Goldman et al. (1981) estimated the smear thickness at 1mm and agreed with previous investigators that it was largely inorganic in composition. They noted its presence along instrumented canal surfaces. Mader et al. (1984) reported that the smear layer thickness was generally 1–2 lm. Cameron (1983) and Mader et al. (1984) discussed the smear material in two parts: first,

superficial smear layer and second, the material packed into the dentinal tubules. Packing of smear debris was present in the tubules to a depth of 40µ. Brannstrom & Johnson (1974) and Mader et al. (1984) concluded that the tubular packing phenomenon was due to the action of burs and instruments. Components of the smear layer can be forced into the dentinal tubules to varying distances (Moodnik et al. 1976, Brannstrom et al. 1980, Cengiz et al. 1990) to form smear plugs (Fig. 2). However, Cengiz et al. (1990) proposed that the penetration of smear material into dentinal tubules could also be caused by capillary action as a result of adhesive forces between the dentinal tubules and the material. This hypothesis of capillary action may explain the packing phenomenon observed by Aktener et al. (1989), who showed that the penetration could increase up to 110 lm when using surface-active reagents in the canal during endodontic instrumentation. The thickness may also depend on the type and sharpness of the cutting instruments and whether the dentine is dry or wet when cut (Barnes 1974, Gilboe et al. 1980, Cameron 1988). In the early stages of instrumentation, the smear layer on the walls of canals can have a relatively high organic content because of necrotic and/or viable pulp tissue in the root canal (Cameron 1988). Increased centrifugal forces resulting from the movement and the proximity of the instrument to the dentine wall formed a thicker layer which was more resistant to removal with chelating agents (Jodaikin & Austin 1981). The amount produced during motorized preparation, as with Gates- Glidden or post drills, has been reported as greater in volume than that produced by hand filing (Czonstkow- sky et al. 1990). However, McComb & Smith (1975) observed under SEM that instrumentation with K-reamers, K-files and Giromatic reciprocating files created similar surfaces. Additional work has shown that the smear layer contains organic and inorganic substances that include fragments of odontoblastic processes, microorganisms and necrotic materials (Pashley 1992). The generation of a smear layer is almost inevitable during root canal instrumentation. Whilst a

noninstrumentation technique has been described for canal preparation without smear formation, efforts rather focus on methods for its removal, such as chemical means and methods such as ultra-sound and hydrodynamic disinfection for its disruption. Root canal preparation without the creation of a smear layer may be possible. A no instrumental hydrodynamic technique may have future potential (Lussi et al. 1993), and sonically driven polymer instruments with tips of variable diameter are reported to disrupt the smear layer in a technique called hydrodynamic disinfection (Ruddle 2007).
When viewed under the SEM, the smear layer often has an amorphous irregular and granular appearance (Bra¨nnstro¨m et al. 1980, Yamada et al. 1983, Pashley et al. 1988) (Fig. 3). The appearance is thought to be formed by translocating and burnishing the superficial components of the dentine walls during treatment (Baumgartner & Mader 1987).

The significance of the smear layer

Root canal treatment usually involves the chemomechanical removal of bacteria and infected dentine from within the root canals. The process is often followed by an intracanal dressing and a root filling. Amongst important factors affecting the prognosis of root canal treatment is the seal created by the filling against the walls of the canal. Considerable effort has been made to understand the effect of the smear layer on the apical and coronal seal (Madison & Krell 1984, Goldberg et al. 1985, 1995, Evans & Simon 1986, Kennedy et al. 1986, Cergneux et al. 1987, Saunders & Saunders 1992, 1994, Gencoglu et al. 1993a, Karagoz-Kucukay & Bayirli 1994, Tidswell et al. 1994, Lloyd et al. 1995, Behrend et al. 1996, Chailertvanitkul et al. 1996, Vassiliadis et al. 1996, Taylor et al. 1997, Timpawat & Sripanaratanakul 1998, Economides et al. 1999, 2004, von Fraunhofer et al. 2000, Froe´s et al. 2000, Goya et al. 2000, Timpawat et al. 2001, Clark-Holke et al. 2003, Cobankara et al. 2004, Park et al. 2004).

Workers have reached different conclusions, with current knowledge of interactions between the smear layer and factors such as filling technique and sealer type being limited. In addition, the methodology of studies, the type and site of leakage tests, and the sample size should be taken into account and consideration given to these variables before conclusions are reached (Shahravan et al. 2007).

Some authors suggest that maintaining the smear layer may block the dentinal tubules and limit bacterial or toxin penetration by altering dentinal permeability (Michelich et al. 1980, Pashley et al. 1981, Safavi et al. 1990). Others believe that the smear layer, being a loosely adherent structure, should be completely removed from the surface of the root canal wall because it can harbour bacteria and provide an avenue for leakage (Mader et al. 1984, Cameron 1987a, Meryon & Brook 1990). It may also limit the effective disinfection of dentinal tubules by preventing sodium hypochlorite, calcium hydroxide and other intracanal medicaments from penetrating the dentinal tubules.

3.2 Should the smear layer be removed?

The question of keeping or removing the smear layer remains controversial (Drake et al. 1994, Shahravan et al. 2007). Some investigations have focussed on its removal (Garberoglio & Brannstrom 1976, Outhwaite et al. 1976, Pashley 1985), whilst others have considered its effects on apical and coronal microleakage (Madison & Krell 1984, Goldberg et al. 1995, Chailertvanitkul et al. 1996), bacterial penetration of the tubules (Pashley 1984, Williams & Goldman 1985, Meryon & Brook 1990) and the adaptation of root canal materials (White et al. 1987, Gencoglu et al. 1993a, Gutmann 1993). In support of its removal are:

1. It has an unpredictable thickness and volume, because a great portion of it consists of water (Cerg- neux et al. 1987).

2. It contains bacteria, their by-products and necrotic tissue (McComb & Smith 1975, Goldberg & Abramovich 1977, Wayman et al. 1979,

Cunningham & Martin 1982, Yamada et al. 1983). Bacteria may survive and multiply (Bra¨ nnstro¨ m & Nyborg 1973) and can proliferate into the dentinal tubules (Olgart et al. 1974, Akpata & Blechman 1982, Williams & Goldman 1985, Meryon et al. 1986, Meryon & Brook 1990), which may serve as a reservoir of microbial irritants (Pashley 1984).

3. It may act as a substrate for bacteria, allowing their deeper penetration in the dentinal tubules (George et al. 2005).

4. It may limit the optimum penetration of disinfecting agents (McComb & Smith 1975, Outhwaite et al. 1976, Goldberg & Abramovich 1977, Wayman et al. 1979, Yamada et al. 1983). Bacteria may be found deep within dentinal tubules (Bystro¨m & Sundqvist 1981, 1983, 1985) and smear layer may block the effects of disinfectants in them (Goldberg & Abramovich 1977, Wayman et al. 1979, Yamada et al. 1983, Baumgart- ner & Mader 1987). Haapasalo & Ørstavik (1987) found that in the absence of smear layer, liquid camphorated monochlorophenol disinfected the den- tinal tubules rapidly and completely, but calcium hydroxide failed to eliminate Enterococcus faecalis even after 7 days of incubation. A subsequent study con- cluded that the smear layer delayed but did not abolish the action of the disinfectant (Ørstavik & Haapasalo 1990). Bra¨nnstro¨ m (1984) had previously stated that following the removal of the smear layer, bacteria in the dentinal tubules can easily be destroyed.

5. It can act as a barrier between filling materials and the canal wall and therefore compromise the formation of a satisfactory seal (Lester & Boyde 1977, White et al. 1984, Cergneux et al. 1987, Czonstkowsky et al. 1990, Foster et al. 1993, Yang & Bae 2002). Lester & Boyde (1977) found that zinc oxide – eugenol based root canal sealers failed to enter dentinal tubules in the presence of smear. In two consecutive studies, White et al. observed that plastic filling materials and sealers penetrated dentinal tubules after removal of smear layer (White et al. 1984, 1987). Oks¸an et al. (1993) also found that smear prevented the penetration of sealers into dentinal tubules,

whilst no penetration of sealer was observed in control groups. Penetration in their smear-free groups ranged from 40 to 60 lm. It may be concluded that such tubular penetration increases the interface between the filling and the dentinal structures, which may improve the ability of a filling material to prevent leakage (White et al. 1984). If the aim is maximum penetration into the dentinal tubules to prevent microleakage, root canal filling materials should be applied to a surface free of smear and either a low surface activity or, alternatively, an adequate surface-active reagent must be added to them (Aktener et al. 1989). However, there are no reports of a correlation between microleakage and penetration offilling materials into dentinal tubules, whilst the basis of leakage studies remains questionable. Pashley et al. (1989) observed an extensive network of microchan- nels around restorations that had been placed in cavities with smear layer. The thickness of these channels was 1–10 lm. Smear layer may thus present a passage for substances to leak around or through its particles at the interface between the filling material and the tooth structure. Pashley & Depew (1986) reported that, when experimenting with class 1 cavi- ties, microleakage decreased after the removal of smear layer, but dentinal permeability increased. Saunders & Saunders (1992) concluded that coronal leakage of root canal fillings was less in smear-free groups than those with a smear layer.

6. It is a loosely adherent structure and a potential avenue for leakage and bacterial contaminant passage between the root canal filling and the dentinal walls (Mader et al. 1984, Cameron 1987b, Meryon & Brook 1990). Its removal would facilitate canal filling (McComb & Smith 1975, Goldman et al. 1981, Cam- eron 1983).

Conversely, some investigators believe in retaining the smear layer during canal preparation, because it can block the dentinal tubules, preventing the ex- change of bacteria and other irritants by altering permeability (Michelich et al. 1980, Pashley et al. 1981, Safavi et al. 1990, Drake et al. 1994, Galvan et al. 1994). The smear layer serves as a barrier to prevent bacterial migration into the dentinal tubules

(Drake et al. 1994, Galvan et al. 1994, Love et al. 1996, Perez et al. 1996). Pashley (1985) suggested that if the canals were inadequately disinfected, or if bacterial contamination occurred after canal preparation, the presence of a smear layer might stop bacterial invasion of the dentinal tubules. Bacteria remaining after canal preparation are sealed into the tubules by the smear layer and subsequent filling materials. Some studies provide evidence to support the hypothesis that the smear layer inhibits bacterial penetration (Pashley et al. 1981, Safavi et al. 1989). A major limitation is that the experiments were undertaken with dentine discs or root cross-sections, models with little relevance in terms of simulating the clinical conditions of root canal treatment. Drake et al. (1994) developed a more clinically relevant model to determine the effect of the presence or absence of the smear layer on bacterial colonization of root canals.

Williams & Goldman (1985) reported that the smear layer was not a complete barrier and could only delay bacterial penetration. In their experiment, using the motile, swarming bacterium Proteus vulgaris, the smear layer delayed the passage of the organisms through the tubules. Madison & Krell (1984) using ethylenediaminetetraacetic acid (EDTA) solution in a dye penetration study found that the smear layer made no difference to leakage. Goldberg et al. (1995) studied the sealing ability of Ketac Endo and Tubliseal in an India ink study with and without smear layer and found no difference. Chailertvanitkul et al. (1996) found no difference in leakage with or without smear layer, however the time period was short. When the smear layer is not removed, the durability of the apical seal should be evaluated over a long period. Since the smear layer is nonhomogeneous and may potentially be dislodged from the underlying tubules (Mader et al. 1984), it may slowly disintegrate, dissolving around a leaking filling material to leave a void between the canal wall and sealer. Meryon & Brook (1990) found the presence of smear layer had no effect on the ability of three oral bacteria to

penetrate dentine discs. All were able to digest the layer, possibly stimulated by the nutrient-rich medium below the discs.

The adaptation of root canal materials to canal walls has been studied. White et al. (1987) found that pHEMA, silicone and Roth 801 and AH26 sealers extended into tubules consistently when smear layer was removed. Gencog lu et al. (1993b) found removing the smear layer enhanced the adaptation of gutta- percha in both cold laterally compacted and thermo- plastic root fillings without sealer. Gutmann (1993) also showed that after removing the smear layer, thermoplastic gutta-percha adapted with or without sealer.

A systematic review and meta-analysis by Shahravan et al. (2007) set out to determine whether smear layer removal reduced leakage of root filled teeth ex vivo. Using 26 eligible papers with 65 comparisons, 54% of the comparisons reported no significant difference, 41% reported in favour of removing the smear layer and 5% reported a difference in favour of keeping it. They concluded that smear layer removal improved the fluid-tight seal of the root canal system, whereas other factors such as filling technique or the type of sealer did not produce significant effects.

Urethane dimethacrylate (UDMA) based root canal sealers have been introduced. Their aim is to provide a better bond to allow less microleakage and increase the fracture resistance of root filled teeth through the creation of monoblocks, when a core material such as Resilon replaces gutta-percha. Whilst some studies indicate that smear layer removal leads to higher

tubule penetration, increased sealer to dentine bond strength and enhanced fluid-tight seal, a recent report concluded that smear layer removal did not necessarily equate to improved resistance to bacterial penetration along these and older types of sealers (Saleh et al. 2008).

Methods to remove the smear layer

Chemical removal

The quantity of smear layer removed by a material is related to its pH and the time of exposure (Morgan & Baumgartner 1997). A number of chemicals have been investigated as irrigants to remove the smear layer. According to Kaufman & Greenberg (1986), a working solution is the one which is used to clean the canal, and an irrigation solution the one which is essential to remove the debris and smear layer created by the instrumentation process. Chlorhexidine, whilst popular as an irrigant and having a long lasting antibacterial effect through adherence to dentine, does not dissolve organic material or remove the smear layer.

Sodium hypochlorite

The ability of NaOCl to dissolve organic tissues is well- known (Rubin et al. 1979, Wayman et al. 1979, Goldman et al. 1982) and increases with rising temperature (Moorer & Wesselink 1982). However, its capacity to remove smear layer from the instrumented root canal walls has been found to be lacking. The conclusion reached by many authors is that the use of NaOCl during or after instrumentation produces superficially clean canal walls with the smear layer present (Baker et al. 1975, Goldman et al. 1981, Berg et al. 1986, Baumgartner & Mader 1987).

Chelating agents

Smear layer components include very small particles with a large surface: mass ratio, which makes them soluble in acids (Pashley 1992). The most common chelating solutions are based on EDTA which reacts with the calcium ions in dentine and forms soluble

calcium chelates (Fig. 4). It has been reported that EDTA decalcified dentine to a depth of 20–30 lm in 5 min (von der Fehr & Nygaard-O¨ stby 1963); however, Fraser (1974) stated that the chelating effect was almost negligible in the apical third of root canals.

Different formulations of EDTA have been used as root canal irrigants. In a combination, urea peroxide is added to encourage debris to float out of the root canal (Stewart et al. 1969). This product (RC-Prep, Premier Dental Products, Plymouth Meeting, PA, USA) also includes a wax that left a residue on the root canal walls despite further instrumentation and irrigation and which may compromise the ability to obtain a hermetic seal (Biesterfeld & Taintor 1980). Many studies have shown that paste-type chelating agents, whilst having a lubricating effect, do not remove the smear layer effectively when compared to liquid EDTA. A recent experiment examining the addition of two surfactants to liquid EDTA did not result in better smear layer removal (Lui et al. 2007).

A quaternary ammonium bromide (cetrimide) has been added to EDTA solutions to reduce surface tension and increase penetrability of the solution (von der Fehr & Nygaard-O¨stby 1963). McComb & Smith (1975) reported that when this combination (REDTA) was used during instrumentation, there was no smear layer remaining except in the apical part of the canal. After using REDTA in vivo, it was shown that the root canal surfaces were uniformly occupied by patent dentinal tubules with very little superficial debris (McComb et al. 1976). When used during and after instrumentation, it was possible to still see remnants of odontoblastic processes within the tubules even though there was no smear layer present (Goldman et al. 1981). Goldberg & Abramovich (1977) observed that the circumpulpal surface had a smooth structure and that the dentinal tubules had a regular circular appearance with the use

of EDTAC (EDTA and cetavlon). The optimal working time of EDTAC was suggested to be 15 min in the root canal and no further chelating action could be expected after this (Goldberg & Spielberg

1982). This study also showed that REDTA was the most efficient irrigating solution for removing smear layer. In a study using a combination of 0.2% EDTA and a surface-active antibacterial solution, Brannstrom et al. (1980) ob- served that this mixture removed most of the smear layer without opening many dentinal tubules or removing peritubular dentine. Bis-dequalinium-acetate (BDA), a dequalinium compound and an oxine derivative has been shown to remove the smear layer throughout the canal, even in the apical third (Kauf- man et al. 1978, Kaufman 1981). BDA is well tolerated by periodontal tissues and has a low surface tension allowing good penetration. It is considered less toxic that NaOCl and can be used as a root canal dressing. A commercial form of BDA called Solvidont (De Trey, A.G., Zurich, Switzerland) was available in the 1980s and its use as an alternative to NaOCl was supported experimentally (Kaufman 1983a,b, Chandler & Lilley 1987, Lilley et al. 1988, Mohd Sulong 1989). Salvizol (Ravens Gmbh, Konstanz, Germany) is a commercial brand of 0.5% BDA and possesses the combined actions of chelation and organic debridement. Kaufman et al. (1978) reported that Salvizol had better cleaning properties than EDTAC. When comparing Salvizol with 5.25% NaOCl, both were found comparable in their ability to remove organic debris, but only Salvizol opened dentinal tubules (Kaufman & Greenberg 1986). Berg et al. (1986) found that Salvizol was less effective at opening dentinal tubules than REDTA.

C ¸alt & Serper (2000) compared the effects of ethylene glycol-bis (ß-aminoethyl ether) tetraacetic acid (EGTA) with EDTA. The smear layer was completely removed by EDTA, but it caused erosion of the peritubular and intertubular dentine, whilst EGTA was not as effective in the apical third of root canals. EGTA is reported to bind calcium more specifically (Schmid & Reilley 1957).

Tetracylines (including tetracycline hydrochloride, minocycline and doxycycline) are antibiotics effective against a wide range of microorganisms. Tetracyclines have unique properties in addition to

their antimicrobial aspect. They have low pH in concentrated solution, and because of this can act as a calcium chelator and cause enamel and root surface demineralization (Bjorvatn 1982). The surface demineralization of dentine is comparable with that of citric acid (Wikesjo¨et al. 1986). Barkhordar et al. (1997) reported that doxycycline hydrochloride (100 mg mL-1) was effective in removing the smear layer from the surface of instrumented canals and root-end cavity preparations. They speculated that a reservoir of active antibacterial agents might remain, because doxycycline readily attaches to dentine and can be subsequently released (Baker et al. 1983, Wikesjo¨et al. 1986). Haznedaroglu & Ersev (2001) showed that 1% tetracycline hydrochloride or 50% citric acid can be used to remove the smear layer from surfaces of root canals. Although they reported no difference between the two groups, it appeared that the tetracycline demineralized less peritubular dentine than the citric acid. In an effort to produce an irrigant capable of both removing the smear layer and disinfecting the root canal system, Torabinejad et al. (2003) developed a new irrigating solution containing a mixture of a tetracycline isomer, an acid, and a detergent (MTAD). Their work concluded MTAD to be an effective solution for the removal of the smear layer. It does not significantly change the structure of the dentinal tubules when the canals are irrigated with sodium hypochlorite and followed with a final rinse of MTAD. This irrigant demineralizes dentine faster than 17% EDTA (De-Deus et al. 2007) and bacterial penetration into filled canals is similar with both solutions (Ghod- dusi et al. 2007).

Organic acids

The effectiveness of citric acid as a root canal irrigant has been demonstrated (Loel 1975, Tidmarsh 1978) and confirmed to be more effective than NaOCl alone in removing the smear layer (Baumgartner et al. 1984). Citric acid removed smear layer better than polyacrylic acid, lactic acid and phosphoric acid but not EDTA

(Meryon et al. 1987). Wayman et al. (1979) showed that canal walls treated with 10%, 25% and 50% citric acid solution were generally free of the smeared appearance, but they had the best results in removing smear layer with sequential use of 10% citric acid solution and 2.5% NaOCl solution, then again followed by a 10% solution of citric acid. However, Yamada et al. (1983) observed that the 25% citric acid–NaOCl group was not as effective as a 17% EDTA–NaOCl combination. To its detriment, citric acid left precipitated crystals in the root canal which might be disadvantageous to the root canal filling. With 50% lactic acid, the canal walls were generally clean, but with openings of dentinal tubules that did not appear to be completely patent (Wayman et al. 1979). Bitter (1989) introduced 25% tannic acid solution as a root canal irrigant and cleanser. Canal walls irrigated with this solution appeared significantly cleaner and smoother than walls treated with a combination of hydrogen peroxide and NaOCl, and the smear layer was removed. Sabbak & Hassanin (1998) refuted these findings and explained that tannic acid increased the cross-linking of exposed collagen with the smear layer and within the matrix of the underlying dentine, therefore increasing organic cohesion to the tubules.

McComb & Smith (1975) compared the efficacy of 20% polyacrylic acid with REDTA and found that it was no better than REDTA in removing or preventing the build-up of smear layer, thought to be as a result of its higher viscosity. McComb et al. (1976) also used 5% and 10% polyacrylic acid as an irrigant and observed that it could remove smear layer in accessible regions. Polyacrylic acid (Durelon liquid and Fuji II liquid) at 40% has been reported to be very effective, and because of its potency users should not exceed a 30 s application (Berry et al. 1987).

Sodium hypochlorite and EDTA

When irrigating a root canal the purpose is twofold: to remove the organic component, the debris originating from pulp tissue and microorganisms, and the mostly inorganic component, the smear layer. As there is no single solution which has the ability to dissolve organic tissues and to demineralize the smear layer, the sequential use of organic and inorganic solvents has been recommended (Koskinen et al. 1980, Yamada et al. 1983, Baumgartner et al. 1984). Numerous authors have agreed that the removal of smear layer as well as soft tissue and debris can be achieved by the alternate use of EDTA and NaOCl (Yamada et al. 1983, White et al. 1984, Baumgartner & Mader 1987, Cengiz et al. 1990). Goldman et al. (1982) examined the effect of various combinations of EDTA and NaOCl, and the most effective final rinse was 10 mL of 17% EDTA followed by 10 mL of 5.25% NaOCl, a finding con- firmed by Yamada et al. (1983). Used in combination with EDTA, NaOCl is inactivated with the EDTA remaining functional for several minutes.

Ultrasonic smear removal

Following the introduction of dental ultrasonic devices in the 1950s, ultrasound was investigated in endodontics (Martin et al. 1980, Cunningham & Martin 1982, Cunningham et al. 1982). A continuous flow of NaOCl activated by an ultrasonic delivery system was used for the preparation and irrigation of canals. Smear-free canal surfaces were observed using this method (Cameron 1983, 1987a,b, Griffiths & Stock 1986, Alacam 1987). Whilst concentrations of 2–4% sodium hypochlorite in combination with ultrasonic energy were able to remove smear layer, lower concentrations of the solutions were unsatisfactory (Cameron 1988). However, Ahmad et al. (1987a) claimed that their technique of modified ultrasonic instrumentation using 1% NaOCl removed the debris and smear layer more effectively

than the technique recommended by Martin & Cunningham (1983). The apical region of the canals showed less debris and smear layer than the coronal aspects, depending on acoustic streaming, which was more intense in magnitude and velocity at the apical regions of the file. Cameron (1983) also compared the effect of different ultrasonic irrigation periods on removing smear layer and found that a 3- and 5-min irrigation produced smear-free canal walls, whilst an 1-min irrigation was ineffective. In contrast to these results, other investigators found ultrasonic preparation un- able to remove smear layer (Cymerman et al. 1983, Baker et al. 1988, Goldberg et al. 1988). Researchers who found the cleaning effects of ultrasonics beneficial used the technique only for the final irrigation of root canal after completion of hand instrumentation (Ahmad et al. 1987a, Alac¸am 1987, Cameron 1988). This is given the term passive ultra- sonic irrigation and has been the subject of a recent review (van der Sluis et al. 2007). Ahmad et al. (1987a,b) claimed that direct physical contact of the file with the canal walls throughout instrumentation reduced acoustic streaming. Acoustic streaming is maximized when the tips of the smaller instruments vibrate freely in a solution. Lumley et al. (1992) recommended that only size 15 files be used to maximize microstreaming for the removal of debris. Prati et al. (1994) also achieved smear layer removal with ultrasonics. Walker & del Rio (1989, 1991) showed no significant difference between tap water and sodium hypochlorite when used with ultrasonics, but they reported that neither solution was effective at any level in the canal to remove the smear layer ultrasonically. Baumgartner & Cuenin (1992) also observed that ultrasonically energized NaOCl, even at full strength, did not remove the smear layer from root canal walls. Guerisoli et al. (2002) evaluated the use of ultrasonics to remove the smear layer and found it necessary to use 15% EDTA with either distilled water or 1% sodium hypochlorite to achieve the desired result.

Laser removal

Lasers can be used to vaporize tissues in the main canal, remove the smear layer and eliminate residual tissue in the apical portion of root canals (Takeda et al. 1998a,b, 1999). The effectiveness of lasers depends on many factors, including the power level, the duration of exposure, the absorption of light in the tissues, the geometry of the root canal and the tip-to-target distance (Dederich et al. 1984, Onal et al. 1993, Tewfik et al. 1993, Moshonov et al. 1995).

Dederich et al. (1984) and Tewfik et al. (1993) used variants of the neodymium–yttrium-aluminium-gar- net (Ne:YAG) laser and reported a range of findings from no change or disruption of the smear layer to actual melting and recrystallization of the dentine. This pattern of dentine disruption was observed in other studies with various lasers, including the carbon dioxide laser (Onal et al. 1993), the argon fluoride excimer laser (Stabholz et al. 1993), and the argon laser (Moshonov et al. 1995, Harashima et al. 1998). Takeda et al. (1998a,b, 1999) using the erbium- yttrium-aluminium-garnet (Er:YAG) laser, demonstrated optimal removal of the smear layer without melting, charring or recrystallization associated with other laser types. Kimura et al. (2002) also demonstrated the removal of the smear layer with an Er:YAG laser. Although they showed removal of the smear layer, photomicrographs showed destruction of peritubular dentine. The main difficulty with laser removal of the smear layer is access to the small canal spaces with the relatively large probes that are available.

Conclusion

Contemporary methods of root canal instrumentation produce a layer of organic and inorganic material called the smear layer that may also contain bacteria and their by-products. This layer covers the instrumented walls and may prevent the penetration of intracanal

medicaments into the dentinal tubules and interfere with the close adaptation of root filling materials to canal walls. The data presented indicate removal of the smear layer for more thorough disinfection of the root canal system and better adaptation of materials to the canal walls. There are, however, no clinical trials to demonstrate this. Current methods of smear layer removal include chemical, ultrasonic and laser techniques – none of which are totally effective throughout the length of all canals or are used universally. However, if the smear layer is to be removed the method of choice seems to be the alternate use of EDTA and sodium hypochlorite solutions. Whilst much is known about individual irrigants, their use in combination and their interactions (and in some cases precipitates) is less well understood. Conflicting reports exist regarding the removal of the smear layer before filling root canals. As several new sealer and core materials have recently been introduced, further investigations are required to determine the role of the smear layer in the outcome of treatment.

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Role of *Enterococcus Faecalis*

Factors that may contribute to a persistent periradicular infection after root canal treatment include intraradicular infection, extraradicular infection, foreign body reaction, and cysts containing cholesterol crystals (1). It is generally believed that the major cause of failure is the survival of microorganisms in the apical portion of the root-filled tooth (1, 2). Unlike primary endodontic infections, which are polymicrobial in nature and dominated by gram-negative anaerobic rods, the microorganisms in- volved in secondary infections are composed of one or a few bacterial species (2–5). Enterococcus faecalis is a persistent organism that, despite making up a small proportion of the flora in untreated canals, plays a major role in the etiology of persistent periradicular lesions after root canal treatment. It is commonly found in a high percentage of root canal failures and it is able to survive in the root canal as a single organism or as a major component of the flora (1). The intent of this article is (a) to describe characteristics inherent to E. faecalis; (b) to cite studies that implicate E. faecalis as an etiology of failing root canal treatment; (c) to list the mechanisms that allow E. faecalis the ability to survive and cause persistent periradicular pathosis; and (d) to discuss current treatment modalities that are effective in eliminating E. faecalis from the root canal system.

4.1 The role of *Enterococcus faecalis* in endodontic infections

Enterococci are gram positive cocci that can occur singly, in pairs, or as short chains. They are facultative anaerobes, possessing the ability to grow in the presence or absence of oxygen (6,7). *Enterococcus* species live in vast quantities [10^5 -10^8 colony-forming units (cfu) per gram of feces] in the human intestinal lumen and under most circumstances cause no harm to their hosts. They are also present in

human female genital tracts and the oral cavity in lesser numbers (8). They catabolize a variety of energy sources including carbohydrates, glycerol, lactate, malate, citrate, arginine, agmatine, and many keto acids (6). *Enterococci* survive very harsh environments including extreme alkaline pH (9.6) and salt concentrations (6, 9). They resist bile salts, detergents, heavy metals, ethanol, azide, and desiccation (6). They can grow in the range of 10 to 45°C and survive a temperature of 60°C for 30 min (9). There are currently 23 *Enterococci* species and these are divided into five groups based on their interaction with mannitol, sorbose, and arginine. *E. faecalis* belongs to the same group as *E. faecium, E. casseliflavus, E. mundtii,* and *E. gallinarum.* These five species form acid in mannitol broth and hydrolyze arginine; however, they fail to form acid in sorbose broth (6, 10). After establishing that the gram-positive coccus is a member of one of the five groups in the *Enterococcus* genus (Table 2) (10), several conventional tests are used to identify the specific species. In group 2, *E. faecalis* can normally be identified by further testing with arabinose, tellurite, and pyruvate. *E. faecalis* is arabinose negative and except for some atypical variants, is the only member of the group to utilize pyruvate and to tolerate tellurite (11). More recently, molecular techniques have been developed that have the capability to rapidly and accurately identify the *Enterococcus* species. Techniques involving DNA-DNA hybridization, sequencing of the 16S rRNA genes, whole-cell protein (WCP) analysis and gas- liquid chromatography of fatty acids have been used for taxonomic purposes. Most of these methods are nucleic acid-based involving PCR amplification assays that are followed by electrophoretic analysis of the PCR products, probing, sequencing, or both (11). Random amplified polymorphic DNA (RAPD) analysis and pulse-field gel electrophoresis (PFGE) are techniques that have been utilized to determine variations in DNA sequences and have been employed in determining various *E. faecalis* subtypes (12, 13). In fact, the Bacteriology Collection of the ATCC (American Type Culture

Collection) currently lists 69 isolates of *E. faecalis* that are commercially avail- able (14). These isolates each have a different ATCC number and designation. The biosafety level ranges from 1 to 2 and growth conditions differ among the subtypes. Sources for these isolates include sour milk (ATCC number 376TM), meat involved in food poisoning (ATCC number 7080TM), and the root canal of a pulpless tooth (ATCC number 4083TM) (14).

Group	Species
Group I (+) acid formation in mannitol broth (+) acid formation in sorbose broth (−) arginine hydrolysis	*E. avium* *E. gilvus* *E. malodoratus* *E. pallens* *E. pseudoavium* *E. raffinosus* *E. saccharolyticus*
Group II (+) acid formation in mannitol broth (−) acid formation in sorbose broth (+) arginine hydrolysis	***E. faecalis*** *E. faecium* *E. casseliflavus* *E. gallinarum* *E. mundtii* *Lactococcus sp.*
Group III (−) acid formation in mannitol broth (−) acid formation in sorbose broth (+) arginine hydrolysis	*E. dispar* *E. durans* *E. hirae* *E. porcinus* *(E. villorum)* *E. ratti*
Group IV (−) acid formation in mannitol broth (−) acid formation in sorbose broth (−) arginine hydrolysis	*E. asini* *E. cecorum* *E. sulfureus*
Group V (+) acid formation in mannitol broth (−) acid formation in sorbose broth (−) arginine hydrolysis	*E. columbae* *Vagococcus sp.*

Table 2. Categorization of Enterococcus species and two physiologically related gram-positive cocci based on phenotypic characteristics

Attention has been turned towards *Enterococci* since the 1970s when they were recognized as major nosocomial pathogens causing bacteremia, endocarditis, bacterial meningitis, urinary tract, and various other infections (15). Sources of the bacteria in these infections have been reported as originating from the hands of health care workers, from clinical instruments, or from patient to patient (8). Studies have shown that nosocomial infections are not caused by the patient's own prehospitalization flora (16). Enterococcal infections

now account for roughly 12% of nosocomial infections in the United States with the majority of those being caused by *E. faecalis* (greater than 80%) and *E. faecium* being responsible for the majority of the remaining infections (17). Studies show *E. faecalis* is able to translocate from the root canal system to the submandibular lymph nodes of germ-free mice, suggesting this route of infection may play a role in the pathogenesis of opportunistic infections in patients (18, 19). Enterococcal urinary tract and soft tissue infections are generally treated with single drug therapy, often with penicillin or vancomycin (20). There is emerging evidence of vancomycin resistance among *Enterococcus* species and routine use of previously standard recommendations for treatment of enterococcal infections can no longer be expected to provide optimal results (21). Enterococcal strains, particularly those causing endocarditis, must be screened to define antimicrobial resistance patterns. Thirty-five vancomycin resistant *Enterococci* have demonstrated susceptibility to linezolid (antibiotic, oxazolidinone derivative), suggesting it may be the treatment of choice for multi-drug resistant enterococcal infections (22).

4.2 Prevalence in Secondary Root Canal Infections

E. faecalis is a normal inhabitant of the oral cavity. The prevalence of *E. faecalis* is increased in oral rinse samples from patients receiving initial endodontic treatment, those midway through treatment, and patients receiving endodontic retreatment when compared to those with no endodontic history (23). *E. faecalis* is associated with different forms of periradicular disease including primary endodontic infections and persistent infections (7). In the category of primary endodontic infections, *E. faecalis* is associated with asymptomatic chronic perira- dicular lesions significantly more often than with acute periradicular periodontitis or acute periradicular abscesses. *E. faecalis* is found in 4 to 40% of primary endodontic infections (7). The frequency of *E. faecalis* found in persistent periradicular lesions has

been shown to be much higher. In fact, failed root canal treatment cases are nine times more likely to contain *E. faecalis* than primary endodontic infections (7). Studies investigating its occurrence in root-filled teeth with periradicular lesions have demonstrated a prevalence ranging from 24 to 77% (3–5, 7, 24–31). The wide range of *E. faecalis* prevalence among studies may be attributed to different identification techniques, geographic differences, or sample size (32, 33). In some cases, *E. faecalis* has been found as the only organism (pure culture) present in root- filled teeth with periradicular lesions (4, 28). The majority of these studies have been carried out using culturing techniques; however, polymerase chain reaction (PCR) is currently a more predictable method for detection of *E. faecalis* (34, 35). This method proves to be faster, more sensitive, and more accurate than culturing methods (35). It has enabled researchers to detect bacteria that were difficult, and in some cases impossible, to detect (35). When compared to detection of *E. faecalis* by culturing (24-70%), *E. faecalis* has been found at consistently higher percentages (67-77%) when a PCR detection method is used (7). An optical spectroscopy-based method has also been studied as a way to detect *E. faecalis* activity (36). It is possible that this detection system could be used chairside to rapidly monitor the presence or absence of *E. faecalis* in the root canal system (36).

4.3 Survival and Virulence Factors

E. faecalis possesses certain virulence factors including lytic enzymes, cytolysin, aggregation substance, pheromones, and lipoteichoic acid (7). It has been shown to adhere to host cells, express proteins that allow it to compete with other bacterial cells, and alter host responses (7, 37). *E. faecalis* is able to suppress the action of lymphocytes, potentially contributing to endodontic failure (38). *E. faecalis* is not limited to its possession of various virulence factors. It is also able to share these virulence traits among species, further contributing to its survival and ability to cause disease (15). These

factors may or may not con- tribute to the innate characteristics of *E. faecalis* to cause disease. Because *E. faecalis* is less dependent upon virulence factors, it relies more upon its ability to survive and persist as a pathogen in the root canals of teeth (7). *E. faecalis* overcomes the challenges of survival within the root canal system in several ways. It has been shown to exhibit widespread genetic polymorphisms (23). It possesses serine protease, gelatinase, and collagen-binding protein (Ace), which help it bind to dentin (39). It is small enough to proficiently invade and live within dentinal tubules (37). It has the capacity to endure prolonged periods of starvation until an adequate nutritional supply becomes available (40). Once available, the starved cells are able to recover by utilizing serum as a nutritional source (40). Serum, which originates from alveolar bone and the periodontal ligament, also helps *E. faecalis* bind to type I collagen (37). *E. faecalis* in dentinal tubules has been shown to resist intracanal dressings of calcium hydroxide for over 10 days (41, 42). *E. faecalis* is able to form a biofilm that helps it resist destruction by enabling the bacteria to become 1000 times more resistant to phagocytosis, antibodies, and antimicrobials than no biofilm producing organisms (43). Calcium hydroxide, a commonly used intracanal medicament, has been shown to be ineffective at killing *E. faecalis* on its own, especially when a high pH is not maintained (42, 44 – 46). The following reasons have been proposed to explain why *E. faecalis* is able to survive intra- canal treatment with calcium hydroxide: (a) *E. faecalis* passively maintains pH homeostasis. This occurs as a result of ions penetrating the cell membrane as well as the cytoplasm's buffering capacity. (b) *E. faecalis* has a proton pump that provides an additional means of maintaining pH homeostasis. This is accomplished by "pumping" protons into the cell to lower the internal pH. (c) At a pH of 11.5 or greater, *E. faecalis* is unable to survive (1, 45). However, as a result of the buffering capacity of dentin, it is very unlikely that a pH of 11.5 can be maintained in the dentinal tubules with current calcium hydroxide utilization techniques (46). Studies using the dentin powder

model have shown that the presence of dentin has an inhibitory effect on various concentrations of root canal medicaments including calcium hydroxide, sodium hypochlorite, chlorhexidine, and iodine potassium iodide (47, 48). Diverse components of dentin including dentin matrix, type-I collagen, hydroxyapatite, and serum are responsible for altering the antibacterial effects of these medicaments (49).

Many studies have been directed towards finding an effective way to eradicate and/or prevent *E. faecalis* from gaining access to the root canal space. *E. faecalis* can gain entry into the root canal system during treatment, between appointments, or even after the treatment has been completed (7). Therefore, it is important to consider treatment regimens aimed at eliminating or preventing the infection of *E. faecalis* during each of these phases. Preparing the apical portion of the root canal to a larger instrument size will help eliminate intracanal microorganisms by reaching areas not normally accessible by smaller master apical files (50). In addition, larger apical preparation sizes facilitate removal of the innermost (pulpal) dentin. This provides the potential to remove intratubular bacteria and open the dentinal tubules to allow antimicrobials to penetrate more effectively. Three percent to full strength sodium hypochlorite, if used in adequate amounts and exchanged regularly, has the capability to destroy *E. faecalis* in the root canal (51). Sodium hypochlorite is an effective irrigant for all presentations of *E. faecalis* including its existence as a biofilm (52). EDTA has little antibacterial activity, but is important in its ability to remove the inorganic portion of the smear layer thus allowing other irrigants access to the dentinal tubules (53, 54). A 10% citric acid solution will remove the smear layer and, like EDTA, has little effect against *E. faecalis*. A 0.1% sodium benzoate solution added to 10% citric acid will increase the chances of killing *E. faecalis* (55). MTAD, a new root canal irrigant consisting of a mixture of a tetracycline isomer, an acid, and a detergent has shown success in its ability to destroy *E. faecalis* in preliminary studies (53,

56). Its effectiveness is attributed to its anticollagenase activity, low pH, and ability to be released gradually over time (56). The effects of MTAD are enhanced when 1.3% sodium hypochlorite is used as an irrigant during instrumentation (57). Calcium hydroxide is relatively ineffective against *E. faecalis* because of considerations mentioned previously (1, 41). Iodine potassium iodide may be a more effective intracanal agent than calcium hydroxide (58). Chlorhexidine, in a 2% gel or liquid concentration, is effective at reducing or completely eliminating *E. faecalis* from the root canal space and dentinal tubules (59 – 61). A 2-min rinse of 2% chlorhexidine liquid can be used to remove *E. faecalis* from the superficial layers of dentinal tubules up to 100 □m (59). Two percent chlorhexidine gel is effective at completely eliminating *E. faecalis* from dentinal tubules for up to 15 days (60). This may be in part attributed to its substantive antimicrobial activity (62). It is questionable as to whether 0.12% chlorhexidine is more effective than calcium hydroxide. Some studies suggest it is more effective, yet neither will completely eradicate *E. faecalis* (44, 63). Another study suggests 10% calcium hydroxide alone is more effective (64). When heated to 46°C, both 0.12% chlorhexidine and 10% calcium hydroxide have greater antimicrobial effects against *E. faecalis* than at normal body temperature (65). Other irrigants that may be effective at eliminating *E. faecalis* include ozonated water and stannous fluoride. Ozonated water has been shown to have the same antimicrobial efficacy as 2.5% sodium hypochlorite (66). Stannous fluoride demonstrated greater antimicrobial effectiveness against *E. faecalis* than calcium hydroxide (67). Combinations of irrigants to eliminate *E. faecalis* have also been studied. In one study, a combination of calcium hydroxide mixed with camphorated paramonochlorophenol completely eliminated *E. faecalis* within dentinal tubules (68). Metapex, a silicone oil-based calcium hydroxide paste containing 38% iodoform, more effectively disinfected dentinal tubules infected with *E. faecalis* than calcium hydroxide alone (69). The addition of stannous fluoride to calcium

hydroxide is also more effective than calcium hydroxide by itself (67). Concentrations of 1 to 2% chlorhexidine combined with calcium hydroxide have also demonstrated efficacy at killing *E. faecalis* (60, 68, 70). Chlorhexidine combined with calcium hydroxide will result in a greater ability to kill *E. faecalis* than calcium hydroxide mixed with water (70). Two percent chlorhexidine gel combined with calcium hydroxide achieves a pH of 12.8 and can eliminate *E. faecalis* within dentinal tubules (60). It is important to note, however, that chlorhexidine alone has been shown to provide as good, or even better, antimicrobial action against *E. faecalis* than calcium hydroxide/chlorhexidine combinations (60, 61). Until further studies have been conducted, an intracanal dressing of 2% chlorhexidine placed for 7 days may be the best way to eradicate *E. faecalis* from dentinal tubules and the root canal space (60, 61). In some studies, chlorhexidine-impregnated and iodoform-containing gutta-percha points have shown little inhibitory action against *E. faecalis* (71, 72). In another study, 5% chlorhexidine in a slow-release device (Activ Point, Roeko, Langenau, Germany) completely eliminated *E. faecalis* in dentinal tubules up to 500 □m (73). The antimicrobial activity against *E. faecalis* of various sealers has also been studied. Roth 811 (Roth International Ltd., Chicago, IL), a zinc-oxide eugenol based sealer, has been shown to exhibit the greatest antimicrobial activity against *E. faecalis* when compared to other sealers (74). AH Plus epoxy-resin based sealer (Dentsply, DeTrey, Konstanz, Germany) and Sultan zinc oxide-eugenol based sealer (Sultan Chemists, Inc., Englewood, NJ) both exhibit good antibacterial effects against *E. faecalis* using agar- diffusion and direct-contact tests (75). AH Plus and Grossman's sealer are effective in killing *E. faecalis* within infected dentinal tubules (76). Based on these studies it can be concluded that a combination of adequate instrumentation, and appropriate use of irrigants, medicaments, and sealer will optimize the chances of eradicating *E. faecalis* during retreatment of failed root canal cases. Additional steps should be taken to prevent *E. faecalis*

from re-entering the root canal space. These include having the patient rinse with chlorhexidine before treatment, disinfecting the tooth and rubber dam with chlorhexidine or sodium hypochlorite, and disinfecting gutta-percha points with sodium hypochlorite before insertion in the canal (77). Other possibilities may include using an obturating system that can provide a more effective seal. Newer obturation systems such as Epiphany (Pentron Corp., Wallingford, CT) have been designed to bond to the root canal walls and thus prevent bacterial leakage. Although research is still needed, a preliminary study shows that this system is better at preventing microleakage of *E. faecalis* than guttapercha filled canals (78). A well-sealed coronal restoration and root canal filling are important steps in preventing bacteria from entering the canal space (79).

Studies indicate that the prevalence of *E. faecalis* is low in primary endodontic infections and high in persistent infections. *E. faecalis* is also more commonly associated with asymptomatic cases than with symptomatic ones. Although *E. faecalis* possesses several virulence factors, its ability to cause periradicular disease stems from its ability to survive the effects of root canal treatment and persist as a pathogen in the root canals and dentinal tubules of teeth. Our challenge as endodontic specialists is to implement methods to effectively eliminate this microorganism during and after root canal treatment. Currently, use of good aseptic technique, increased apical preparation sizes, and inclusion of full strength sodium hypochlorite and 2% chlorhexidine irrigants are the most effective methods to eliminate *E. faecalis*. Recent studies have helped us better understand *E. faecalis* and the mechanisms that enable it to cause persistent endodontic infections. In the changing face of dental care, continued research on *E. faecalis* and its elimination from the dental apparatus may well define the future of the endodontic specialty.

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5. The presence of Enterococcus faecalis in saliva as a risk factor for endodontic infection.

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Introduction

The role of bacteria in the initiation and progression of endodontic infections has been widely demonstrated[1]. Bacteria can invade the root canal system *via* different pathways such as carious and periodontal lesions, cracks or, less commonly, blood stream[2]. Profiling of endodontic infections revealed compositionally unspecific, yet differentially abundant microbiota depending on clinical diagnosis[3]. Meanwhile, primary infections are caused by microorganisms which initially invade and colonize necrotic root canals, secondary and persistent infections are caused by microorganisms introduced into root canals secondary to professional intervention or survive the chemo-mechanical debridement and persist within the root canal environment[4]. *Enterococcus faecalis* is an anaerobic gram-positive bacterium which has been frequently recovered from secondary/persistent endodontic infections[5,6]. The contribution of *E. faecalis* to endodontic treatment failures is attributed to its ability to withstand nutrient scarcity encountered in root-filled teeth[7,8], and tolerance to antimicrobials implemented during endodontic treatment[9,10]. The ability of *E. faecalis* to form dense biofilms on root canal walls, and to

invade dentinal tubules and root canal complexities contribute to its recalcitrance to endodontic disinfectants and intracanal dressings [11,12,13]. These attributes contribute to persistence and recovery of *E. faecalis* from endodontic failures [14,15]. Given that endodontic microbiota is derived from oral microbiota under the influence of specific ecological conditions of root canal environment [4], and despite the recovery of *E. faecalis* from root-filled teeth with post-treatment diseases, *E. faecalis* is not a typical member of commensal oral microbiota[15]. It is less likely that *E. faecalis* occurs in advanced carious lesions, and primary endodontic infections[16]. Therefore, the origin of *E. faecalis* recovered from root canals has been questioned, and its association with their respective counterparts in saliva was studied17. A significant association was found between the presence of *E. faecalis* in saliva and root canals with post-treatment apical periodontitis[18]. Similar genotype was detected in *E. faecalis* isolated from saliva and endodontically treated teeth[19], while different genetic profiles were observed in salivary and root canals strains from the same patient[20]. Therefore, the origin of *E. faecalis* in endodontic treatment failures was proposed to be exogenous[21]. With such contradictory findings, the relationship between *E. faecalis* in saliva and root canals remains unsolved and additional evidence is warranted. Therefore, the aim of this study was to determine the prevalence of *E. faecalis* in root canals and saliva and to investigate whether its presence could influence the presence and dimension of periapical lesions.

Materials and Methods

Study design

The present cohort study is reported following the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) guidelines for cohort studies[22]. The research protocol was approved by the local Ethics Committee (protocol number: 18202/2020) and was registered on Clinicaltrials.gov (NCT04637659).

Setting and participants

Sixty-seven patients were sequentially recruited among those attending the Unit of Endodontology and Restorative dentistry, School of Dentistry, University of Siena between July 2020 and November 2020 according to the following eligibility criteria:

- need for a root canal treatment or retreatment with previous therapy aging for at least five years.
- ability and willingness to give informed consent.

The exclusion criteria were:

- presence of periodontitis[23].
- impossibility to isolate the operating field.
- retreatment cases with missing or calcified canals, perforation and separated endodontic instruments in which was impossible to reach the apex.
- administration of antibiotics within the last 3 months.
- patients with diabetes, rheumatoid arthritis, and inflammatory bowel diseases.

The cohort of patients included in the present study was defined once all participants read and signed a written informed consent, according to the Declaration of Helsinki.

Variables

Clinical and radiographic assessment

For each participant, demographic characteristics (age, gender) as well as medical and dental history were collected. Tooth position (anterior/posterior) and type of coronal restoration (direct/indirect) were recorded during the clinical examination; The quality of each restoration was defined as proper or improper, according to the "Modified USPHS " criteria[24]. A standardized periapical intraoral radiograph was performed to evaluate the status through the Periapical Index (PAI) score[25]. Afterwards, the included teeth were categorized into five groups according to their pulpal and periapical status as determined by clinical and radiographic findings[27]: i) healthy vital tooth (HVT) and ii) healthy treated tooth (HTT) being treated or retreated for restorative or prosthodontic reasons; iii) irreversible pulpitis (IP); iv) pulp necrosis (N); post-treatment apical periodontitis (PTAP).

Sampling and clinical procedures

Root canal and saliva samples were collected as previously described [27]. Before isolation with the rubber dam, saliva samples from the floor of the mouth, dorsum of the tongue and the crown of the affected tooth were collected for each patient using three sterile ISO size 40 paper points (Dentsply-Maillefer, Ballaigues, Switzerland). The paper points were resuspended in 100 µl of PBS/10% glycerol and stored at -70°C until analysis. Plaque around the affected tooth was removed using scalers and the surfaces were brushed with pumice. Teeth were isolated with a rubber dam, and disinfected with 30% hydrogen peroxide and 5.25 % sodium hypochlorite

(NaOCl), which is inactivated by sodium thiosulphate 5%. As a sterility control, three sterile paper points (Size 40) were rubbed on the crown of the tooth and on the surrounding areas. After access preparation, root canal patency was achieved with minimal instrumentation and without using hypochlorite irrigant. In case of retreatment, coronal gutta percha was removed by sterile Gates Glidden drills size 2 & 3 (Dentsply-Maillefer, Ballaigues, Switzerland), while the middle and apical gutta percha were removed with endodontic files without a chemical solvent. Irrigation was performed with sterile saline to remove any residual material before the collection of the intracanal sample. Once the working length was established, the pre-treatment sample was collected using ISO size 10 K-file (Dentsply-Maillefer, Ballaigues, Switzerland). An additional pretreatment sampling was performed by introducing two sterile paper points (ISO size 15) into the full working length kept for at least 60 seconds. The sample was then transferred to PBS/10% glycerol solution. When the canal was dry, a sterile paper point moistened with sterile saline was used to acquire the sample. In multi-rooted teeth, a single root canal was chosen, based on the presence of periapical radiolucency and/or exudation.

Laboratory assessment

Isolation and identification of Enterococci

Ten µl of PBS/10% glycerol from each sample were plated on Brain Heart Infusion (BHI) agar containing 5% horse blood. The plates were incubated in

5% CO_2 at 37°C for 48 hours, and monitored daily for the presence of microbial growth. Putative enterococcal colonies were isolated on BHI agar/blood and identified with a latex agglutination test (Oxoid™ Streptococcal Grouping Kit, Thermo Fisher, Hampshire, United Kingdom). Group D colonies were then identified on a MALDI Biotyper (Bruker Daltonics, Bremen, Germany) and by ribosomal RNA operon sequencing [28]. Colonies identified as *E. faecalis* were frozen at -70°C in BHI/10% glycerol.

High molecular weight DNA extraction

E. faecalis strains were streak plated on BHI agar/blood, incubated overnight at 37°C and checked for purity. About ten single colonies were inoculated in BHI broth and the starter cultures of exponentially growing bacteria (OD590 of 0.3-0.4) were frozen at -70°C with 10% glycerol. Bacteria were inoculated 1:50 (vol:vol) from starter cultures in 10 ml of BHI broth and incubated at 37° C until an OD_{590} of 1.0 was reached. Samples were then centrifuged at 6600 x *g* for 5 minutes. Bacterial pellets were washed with 10 ml of sterile 1X TE buffer (Tris 10 mM-EDTA 1 mM) and resuspended in 7.5 ml of Raffinose buffer (50 mM Tris pH 8, 5 mM EDTA, 20 % Raffinose). DNA extraction was carried out as described previously [29]. The DNA pellet was resuspended in 100 µl of saline. Genomic DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen, Whaltan, Massachusetts, USA) and a NanoPhotometer device (Implen, Westlake Village, USA) before molecular analysis and whole genome sequencing.

Sequencing and bioinformatic analysis

Whole genome sequencing (WGS) was performed employing Oxford Nanopore technology. Following manufacturers' instruction, the sequencing library was prepared using a ligation sequencing kit (SQK-LSK108) and barcode expansion kits (EXP-NBD104/114) for sample multiplexing. The sequencing run was performed on the GridION x5 platform (Oxford Nanopore Technologies). Samples were also sequenced with Illumina technology at MicrobesNG (Birmingham, UK) (https://microbesng.com/) which performed library preparation and sequencing of paired end 250 bp reads on a HiSeq2500. Genomes were *de novo* assembled using Unicycler (v 0.4.7)[30], with both Nanopore and Illumina reads as an input. Phylogenetic relationships among sequenced genomes were explored using PopPUNK (v. 2.4.0) using the 'fit-model lineage' parameter for data fitting[31]. PopPUNK exploits the Jaccard index (J) to establish the similarity between k-mer data sets (oligonucleotide sequences of k length) of two genome sequences ($0<J<1$, with $J=1$ describing two genome sequences sharing the same k-mers)[32].

Power analysis

The detection rate of *E. faecalis* in culture medium was reported to be 2% and 71% in primary and secondary endodontic infections respectively[33]. Therefore, setting the level of significance at alpha=0.05, the power of the study resulted to be above 90%.

Statistical analysis

All analyses were performed using a statistical software (STATA BE, version 17.1, StataCorp LP, Texas, USA), setting the level of significance at 5%.

Continuous variables were expressed as Mean (SD), while categorical variables were expressed as number of observations (percentage - %). The fisher's exact test was used to investigate the association between clinical and microbiological variables. Simple linear/logistic multilevel regression models were built in order to evaluate the association between *E.faecalis* presence in the canal before treatment/ E.faecalis presence in saliva and tooth vitality, presence of periapical lesion and PAI score, respectively. Multiple multilevel regression models were obtained by adjusting the crude estimates for confounders (i.e. proper/improper restoration, type of restoration, tooth position).

Results

Participants and samples

Sixty-seven patients (36 males and 31 females), aged from 26-90 (mean ± SD = 56 ± 1.67, 95% CI: 52.36%, 59.03) were included in the study. A total of 79 teeth in the recruited patients were sampled. Eleven samples were discarded due to sampling or laboratory errors, and one saliva sample was repeated in the same patient after 4 months after endodontic therapy. Therefore, a total of 67 teeth were included in the final analysis. *E. faecalis* was recovered from 11 (16.42%) and 7 (10.45%) root canal and saliva samples, respectively. The highest frequency was from the PTAP group (30%), followed by N (22.2%) and HTT (16.6%) groups. *E. faecalis* was not detected in IP and HVT groups. Descriptive statistics of patients' characteristics, and clinical and microbiological assessments are shown in Table 1.

Outcome data

Clinical and microbiological variables

Results of the association between clinical and microbiological variables are shown in Table 2. The pretreatment presence of *E. faecalis* in root canals was significantly associated with the presence in saliva ($p<0.001$) and the presence of periapical lesions ($p<0.05$) . The tooth position, type and quality of coronal restorations were not significantly associated with the presence of *E. faecalis* in any of the samples.

Linear/logistic regression analyses

The combined effect of the variables that were related to the presence of *E.faecalis* in precanal samples were investigated using a logistic regression model (Table 3). The presence of *E. faecalis* in root canal samples before the treatment significantly increased the odds of having a secondary endodontic infections (OR=2.94; 95% CI [1.47, 11.59]; $p<0.05$) while its presence in saliva was associated with higher odds of identifying *E. faecalis* in root canals (OR=3.70; 95% CI [1.031, 19.229]; $p<0.05$) and to develop a secondary/persistent infection (OR=3.07; 95% CI [1.67, 6.88]; $p<0.05$). The presence of *Enterococcus faecalis* in root canals significantly increased the odds of periapical lesion (OR=11.03; 95% CI [1.273, 95.704]; $p<0.05$). However, this was not the case when *E. faecalis* was identified in saliva (OR=1.97; 95% CI [0.333, 11.674]; $p<0.454$). Finally, the presence of *Enterococcus faecalis* in pretreatment samples increases the odds of a higher PAI index score (MD=1.031; 95% CI[0.091, 1.971]; $p<0.05$).

Phylogenetic relationships of E. faecalis isolates

The phylogenetic relationships among different isolates were investigated using PopPUNK, as deduced from the whole genome sequences (Figure 1). Core genome analysis and whole genome analysis identified five major clusters. Analysis of phylogenetic distances among isolates using the Jaccard index with different k-mer lengths indicated that saliva and root canal isolates of *E. faecalis* retrieved from the same patient, BE15 and BE43, BE16 and BE17, BE7 and BE8, BE32 and BE33 were highly correlated (J>0.95). Moreover, the strains BE11 and BE32 were recovered from the saliva of the same subjects at 2 different visits 4 months apart, and share a J index of 0.987 (k=29). The strains BE5 and BE52 share a J index of 0.8 although they were recovered from root canals of different subjects.

Discussion

The present study aimed to assess the prevalence and correlation between *E. faecalis* isolates from root canals, with different pulpal and periapical conditions, and saliva to better understand the origin of *E. faecalis* in endodontic infections. This study analyzed the association of *E. faecalis* presence in root canals before treatment with (i) the status of periapical tissues, and (ii) clinical characteristics such as type, quality and location of restorations. Previous studies investigated the prevalence of *E. faecalis* in failed endodontic treatment and persistent infections[18,19,20], and recovered *E. faecalis* from primary endodontic infections[5,,27,,34]. This study included clinical conditions ranging from healthy pulp to teeth with post-treatment apical periodontitis, classifying each condition into primary or

secondary/persistent endodontic infection groups as previously established[36]. A cultural approach was used to isolate *E. faecalis* from saliva and endodontic samples, this allowed to recover strains for further molecular characterization and to avoid PCR-based techniques, which could be influenced by contamination and by the presence of extracellular DNA or DNA from dead bacterial cells[37,38]. Recently, Next Generation Sequencing (NGS)-based studies revealed an unspecific composition of endodontic microbiota[4], and challenged the role of *E. faecalis* in the etiology of persistent/secondary root canal infections [39,,40]. *E. faecalis* was not identified in root canals with healthy vital pulp or irreversible pulpitis, coherently with the reported absence of *E. faecalis* in carious lesions close to the pulp[16]. On the other hand, a more recent NGS-based study identified the genus *Enterococcus* in the microbiome of root canals with irreversible pulpitis, albeit at a very low relative abundance[41]. According to our study, the prevalence of *E. faecalis* in necrotic root canals was 22%. This percentage was essentially in line with a previous study, wherein the prevalence of *E. faecalis* was 26% and 32% when identified by culture- and PCR-based methods, respectively[42]. The prevalence of *E. faecalis* in primary root canal infections was even lower (7.5%) when investigated using the checkerboard DNA-DNA hybridization[43]. These findings collectively support a relatively low occurrence of enterococci in primary endodontic infections. This could be explained by the fact that enterococci are transient members of oral microbiota[18], given that endodontic microbiota are derived from oral microflora influenced of the specific ecological conditions of root canal system[44]. It is also possible that microbial species predominant in primary endodontic infections can inhibit the

proliferation of *E. faecalis*, yet such assumption should be investigated in the future studies. Our study revealed that *E. faecalis* was identified by 30% in secondary/persistent endodontic infections, which lies in agreement with previous sequencing-based studies, which reported a prevalence of *E. faecalis* equal or greater than 30% in these infections[45,46]. The higher prevalence of *E. faecalis* in secondary/persistent endodontic infections group compared to primary infections in this study (30% vs 22%) agrees with a previous systematic review, which significantly correlated *E. faecalis* with persistent infections[13]. Adaptation to environmental conditions of root-filled teeth and tolerance to intracanal disinfection could explain the higher occurrence of *E. faecalis* in post-treatment apical periodontitis[8]. It has been demonstrated that mechanical instrumentation and exposure to endodontic irrigants increased the number and adhesion forces of *E. faecalis* to dentine and root canal filling materials respectively[47,48,49]. Our study reported a 10% prevalence of *E. faecalis* in saliva samples. Previous studies, both using cultural and molecular methods, reported similar values of prevalence ranging from 19 to 21%[18,19]. Isolation of *E. faecalis* from saliva samples could be also linked with the isolation of this pathogen from multiple oral sites[19], which supports the assumption that oral cavity could be a potential reservoir of *E. faecalis*. In contrast to our and previous studies, *E. faecalis* was never identified in saliva of patients seeking endodontic retreatment[20]. In addition, a significant association was observed between the presence of *E. faecalis* in saliva and root canals, as demonstrated previously[18], while contradicting an earlier study[21]. The higher odds of identifying *E. faecalis* in root canals when it exists in saliva supports a possible role of *E. faecalis* in saliva as a risk factor for

root canal infection with this pathogen. A higher prevalence of *E. faecalis* in saliva and subgingival samples from patients with chronic periodontitis compared to healthy subjects was reported[50], and suggests that periodontal infections could favor the colonization of *E. faecalis* as observed in endodontic-periodontal lesions[35]. For this reason, in our study, subjects with periodontitis were excluded. Our study also agrees with the study by Wang et al.[19], wherein tooth position, quality and type of restorations were not significantly associated with the presence of *E. faecalis* in root canals despite differences in the demographic characteristics of the investigated populations. Our results demonstrated that the odds of developing a periapical lesion were significantly increased when *E. faecalis* was detected in root canals. These results could be explained by several studies, which demonstrated the role of *E. faecalis* and its virulence factors (such as extracellular proteases and cytolysin) in local inflammation and alveolar bone destruction in apical periodontitis [51,52]. Our results correlate with the study by Molander et al., wherein enterococci were recovered from 32% of teeth with radiographically verified apical periodontitis versus only 5% in teeth with no apical periodontitis[53], while other studies revealed no significant association of enterococci with diseased periapical tissues[54,55]. Although it is well-established that AP is of bacterial etiology, it is important to consider that multiple local and systemic factors predispose the incidence of periapical lesions and affect the healing of periapical tissues in endodontically-treated teeth[56,,57]. Our pilot findings showed that two genetically related salivary isolates of *E. faecalis* were recovered from the same subjects at four months apart, which could support the assumption that this species could persist in

the oral cavity for a long time frame, as observed in repeated oral rinses after the ingestion of enterococci-rich food and in mature biofilms recovered from intraoral dental splints[58,59]. We demonstrated genetic relatedness of four pairs of salivary and endodontic *E. faecalis* isolates from the same patient, this supports the hypothesis that *E. faecalis* in saliva could serve as a potential source of infecting root canals. A similar finding was also reported for *E. faecalis* strains isolated from saliva, pulp chamber and root canals of endodontic patients[20]. These findings can be explained by the possible transition of *E. faecalis* from oral cavity into root canals during or after endodontic treatment or less likely *via* carious lesions. We also found a pair of genetically different *E. faecalis* in saliva and root canals of the same patient (BE51 and BE52). Interestingly, strain BE52 was genetically related to BE5, which was isolated from the root canal of a different patient. These findings suggest that similar strains of E. faecalis can be present in different individuals as observed by Pinheiro et al.[60], which could be related to bacterial intake by exogenous sources such as food[17]. The future studies should be focused on investigating the genetic profiles of *E. faecalis* strains longitudinally collected from the same patient, and their association with food intake. It seems also worthy to explore the long-term occurrence of *E. faecalis* in the oral cavity in a larger cohort, and to investigate the factors which govern the long-term survival of *E. faecalis* and its integration into oral biofilms. The mechanisms which explore the role of *E. faecalis* in the pathogenesis of AP should also be investigated.

Conclusion

The findings of this study confirmed the presence of *E. faecalis* in saliva and root canals especially those with post-treatment apical periodontitis. The significant association and genetic relatedness of *E. faecalis* in saliva and root canals suggest that the presence of *E. faecalis* in saliva is a risk factor for root canal contamination with this pathogen. The latter could increase the risk of developing a periapical lesion. The present study shifts the focus back to the role of *E. faecalis* in the pathogenesis of endodontic infections.

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Table 1. Descriptive statistics of patients' characteristics.

Variable		Mean±SD/Proportion (%)
Age		55.69±1.67
Gender		
	Females	31 (45.56%)
	Male	36 (54.44%)
Groups		
	N	18 (26.87%)
	HVT	7 (10.45%)
	IP	13 (19.40%)
	PTAP	20 (29.85%)
	HTT	9 (13.43)%
Lesion		
	Present	49 (73.13%)
	Absent	18 (26.87%)
PAI score		
	0	18 (26.87%)
	1	16 (23.88%)
	2	7 (10.45%)
	3	20 (29.85%)
	4	4 (5.97%)
	5	2 (2.99%)
Position		
	Anterior	38.81%
	Posterior	61.19%
Restoration type		
	Indirect	46 (68.66%)
	Direct	21 (31.34%)
Saliva		
	Present	7 (10.45%)
	Absent	60 (89.55%)
Canal pre-treatment		
	Present	16.42%
	Absent	83.58%

Abbreviations: SD, standard deviation; N, necrotic tooth; HVT, healthy vital tooth; IP, irreversible pulpitis; PTAP, post-treatment apical periodontitis; HTT, healthy treated tooth; PAI score, periapical index score. Saliva +, proportion of saliva samples positive for *E. faecalis*; Canal pre-treatment, proportion of samples in the canal before treatment positive for *E. faecalis;* Dash symbol, not measured.

Variable/ *E. faecalis* in sample	Saliva (yes/no)	p value	PreCanal (yes/no)	p value
Group				
N	5/13		3/15	
HVT	0/7	0.103	0/7	0.139
IP	0/13		0/13	
PTAP	2/18		6/14	
HTT	0/9		2/7	
Lesion				
present	6/49	0.665	11/49	***0.023****
absent	1/18		0/18	
PAI score				
0	1/18		0/18	
1	0/16	0.071	3/13	***0.030****
2	0/7		0/7	
3	4/20		6/14	
4	1/4		2/4	
5	1/2		0/2	
Quality of restoration				
Proper	3/46	0.132	0/10	0.195
Improper	4/21		11/57	
Type of restoration				

Direct	1/10	0.721	7/46	0.730
Indirect	6/57		4/21	
Position				
Anterior	4/26	0.257	5/26	0.738
Posterior	3/41		6/41	
Precanal sample				
present	5/7	***0.001***	—	—
absent	6/60		—	

Table 2. inferential statistics and Fisher's test values.

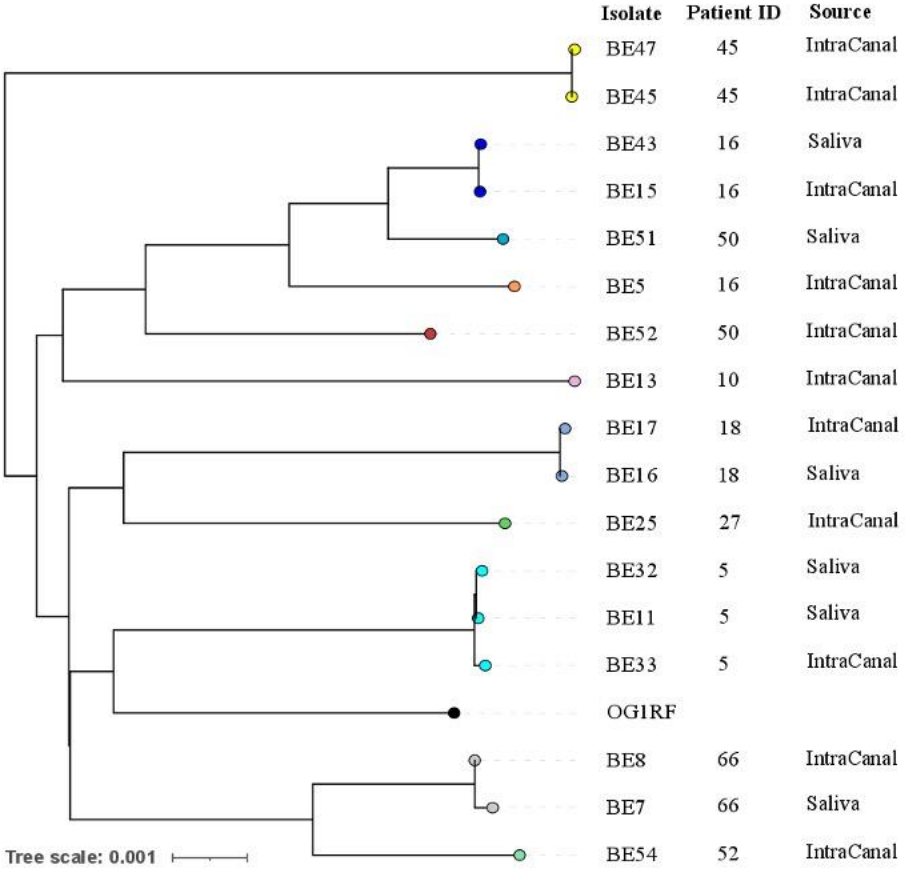
Abbreviations: N, necrotic tooth; HVT, healthy vital tooth; IP, irreversible pulpitis; PTAP, post-treatment apical periodontitis; HTT, Healthy treated teeth; Dash symbol, not measured; PAI score, periapical index score; Saliva +, proportion of saliva samples positive to *E. faecalis*; Precanal sample, proportion of samples in the canal before treatment positive to *E. faecalis*; proportion of samples in the canal after treatment positive to *E .faecalis*. **p*<0.05,***p*<0.001.

Table 3. Linear/logistic regression analyses for the association between *E.faecalis* in the root canal/saliva and PAI score with clinical variables, respectively.

Variable	**Presence of *Enterococcus faecalis* in root canal before treatment**									
	N	**Crude ORs**	**95% CI**		***p*-value***	**N**	**Adjusted † ORs**	**95% CI**		***p*-value***
			Lower	**Upper**				**Lower**	**Upper**	
Secondary infection groups	67	3.6	1.5	13.63.1	***0.049****	57	2.95	1.5	11.6	***0.023****
Presence of periapical lesion	67	9.3	77.6	12.4	***0.039****	57	11.0	1.2	95.7	***0.029****
	Presence of *Enterococcus faecalis* in saliva									
Secondary infection groups	67	0.9	0.16	5.26	0.938	67	3.07	1.67	6.88	***0.040****
Presence of periapical lesion	67	1.9	0.3	10.6	0.460	67	1.9	0.3	11.6	0.454
	PAI score									
	N	**Crude MD**	**95% CI**		***p*-value***	**N**	**Adjusted † MD**	**95% CI**		***p*-value***
			Lower	**Upper**				**Lower**	**Upper**	
Enterococcus faecalis in precanal samples	67	1.08	0.1	2.0	***0.022****	67	1.03	0.1	1.9	***0.032****

Abbreviations: ORs, odds ratios; CI, confidence interval; secondary infection group (PTAP,HTT). * $p<0.05$, †Adjusted for tooth position, type and proper/improper restoration.

Figure 1: Phylogenetic relationships among *E. faecalis* isolates. Different colors indicate different genetic clusters. The phylogenetic tree was generated based on whole genome sequences with branch lengths indicating the number of nucleotide substitutions per site (scale bar), whereas major five population clusters (coloured dots) were obtained. Other closed clusters, by different patient samples, were identified, in particular between the strains BE 15 and BE 43, BE 15 and BE 51 (J>85%), BE 7 and BE 54, BE 5 and BE 52 (J>80%), BE 16 and BE 25 (J>60%).


Isolate
Patient ID
Source
BE47 45 IntraCanal
BE45 45 IntraCanal
BE43 16 Saliva
BE15 16 IntraCanal
BE51 50 Saliva
BE5 16 IntraCanal
BE52 50 IntraCanal
BE13 10 IntraCanal
BE17 18 IntraCanal
BE16 18 Saliva
BE25 27 IntraCanal
BE32 5 Saliva
BE11 5 Saliva
BE33 5 IntraCanal
OG1RF
BE8 66 IntraCanal
BE7 66 Saliva
BE54 52 IntraCanal
Tree scale: 0.001

3

3. Influence of methodological variables on fracture strength tests results of premolars with different number of residual walls. A systematic review with meta-analysis.

Gaeta C, Marruganti C, Mignosa E, Franciosi G, Ferrari E, Grandini S.
Dent J (Basel). 2021 Dec 2;9(12):146.

1 Introduction

Restorative procedures and Endodontic treatment produce extensive loss of dentine structure favoring risk of fracture and tooth loss[1,2]. It is widely accepted that the extension of access cavity during endodontic treatment and consequently the number of walls lost as well as the removal of occlusal marginal ridges could sensibly affect the strength of the teeth involved. Upper premolars are more prone to fracture compared to molars because of their position in the mouth and the anatomical features, such as the shape, crown volume and crown/root proportion[3]. Fracture strength test remains a common experimental method to evaluate the restorative procedure for root filled teeth despite shortcomings are highlighted regarding the correct physiological load, the teeth used in the experiment, and the differences in test conditions[4]. The pattern of loading plays a crucial role in the fracture strength test, in which it tends to simulate the occlusal forces in the mouth. The direction and location of the loading tip as well as the shape and the diameter may influence the results of the test. Usually, the direction of the applied forces, used in this in vitro test, are axial on both buccal and palatal cusps with an inclination of 30/45°, and this could sensibly influence the outcome of the test[5]. The periodontal ligament is an

anatomical structure able to distribute the occlusal load thanks to its fibers. Its inclusion in the fracture strength test is confirmed by a finite element analysis study[6] and it seems to influence the results of the in vitro test[7]. Thermo cycling is an in vitro procedure used to simulate the thermal stresses that usually occur during masticatory function[8]. This tooth aging treatment seems to considerably affect the results, if applied[9]. To date, no systematic review or meta-analysis is present regarding the impact of methodological variables related to the experiment on the fracture strength of the teeth evaluated. Such results may have an implication regarding the testing phase of different restorative materials, and could also serve as a guide for future studies. The aim of the present systematic review is to evaluate the influence of methodological variables such as Thermocycling, Periodontal ligament simulation, Load inclination and Tip diameter, on fracture strength test of upper premolar with intact, 0,1,2 walls lost.

2 Materials and Methods

2.1 Protocol and registration

The present systematic review was performed following the PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) statement [1].

2.2 Eligibility criteria

All *ex vivo* studies performing a fracture strength test on premolars were included in the present protocol. Inclusion criteria were defined with the PICO(S) method:

- Population: intact premolars or with 0/1/2 walls lost;
- Intervention: fracture strength test;
- Comparison: no comparison group was defined;

- Outcome: fracture strength, measured in Newton;

- Studies: all ex vivo studies.

2.2.1 Exclusion criteria

All studies where an indirect restoration, fiber post or endocrown were performed, were excluded from the current protocol.

2.3 Information sources

The search strategy included the screening of electronic databases and the relevant journals. Other sources encompassed citations from relevant articles. The screening and inclusion stages were reported following the PRISMA flow diagram (Fig. 1).

2.3.1 Electronic search

The electronic databases screened were MEDLINE (through Pubmed) and Google Scholar; a search for grey literature was also carried out (OpenGrey). A combination of MeSH terms and free text words was used to define the full electronic strategy. Only studies published in English or Italian were included; no restrictions as to publication date were applied.

The full electronic search strategy is reported for MEDLINE (through PubMed): Fracture Strength Test AND (endodontics OR endodontically treated teeth).

The search strategy was then adapted for the other databases.

2.3.2 Hand search

Relevant journals (Journal of Endodontics, International Journal of Endodontics, Journal of Prosthetic Dentistry) were screened by two independent reviewers (C.M., C.G.), assessing all issues from January 1985 until June 2021. Other sources encompass narrative reviews and references from relevant articles.

2.4 Study selection

Titles and abstracts were independently screened for relevance by two calibrated reviewers (un-weighted Cohen's *k* score of 0.90) (C.M., C.G.). Subsequently, the pre-selected articles were screened for full-text analysis by both reviewers according to the eligibility criteria. Any disagreement at any stage (title/abstract or full text) was resolved through discussion with a third author (S.G.) in order to reach consensus.

2.5 Data collection process

Data collection was performed through an extraction sheet by two independent reviewers (C.M., C.G.) during full-text analysis. Characteristics of the included studies encompassed: fracture strength test of intact upper premolars or with, 0, 1 and 2 walls lost.

2.6 Data items

The primary outcome of the protocol was defined as the maximum fracture strength value expressed in Newton (Kg x m/s^2). The methodological variables such as PDL simulation, inclination of the load, tip position and diameter, thermocycling were registered and used to perform subgroup analysis and meta-regression.

2.7 Summary measures

In the present systematic review, fracture strength values were considered as the main outcome and a precomputed effect size with 95% Confidence Interval was calculated. Whenever it was not available, fracture strength was calculated through raw data analysis.

2.8 Synthesis of results and additional analyses

Statistical analysis was carried out through an *ad hoc* statistical software (STATA BE, version 17, StataCorp LP, College Station, TX, USA).

Cochran's Q statistic and the I^2 index were used in order to estimate heterogeneity across studies [4,5]. Between-study variance was estimated with the T^2 parameter. Meta-analysis of the included studies was conducted through an inverse variance analysis using the Der Simonian and Laird [6] random effects model. The analysis was performed using the pre-computed effect size pooled for each study. Subgroup analysis was performed (for each group: intact premolars, 0,1,2 walls lost) about the following variables:

- *Periodontal ligament simulation*;
- *Load inclination*;
- *Tip position*;
- *Thermocycling*;

Meta-regression of the covariate *Tip diameter* was also performed. The obtained results were graphically showed by *forest plot*. Moreover, publication bias was

assessed through the Egger's test [7]. Values of $p<0.05$ were considered statistically significant.

3 Results

3.1 Study Selection

Initial electronic search retrieved a total of 459 articles in MEDLINE. Manual search in relevant journals led to no additional records. 31 were excluded because of the time of publication. After the exclusion of 294 records at the eligibility level, 134 were assessed for full-text eligibility. Finally, 25 studies were included in both the qualitative and quantitative (meta-analysis) synthesis of data, from which 17 subgroups were extrapolated for Intact premolar, 17, 6, 45 respectively for 0, 1, 2 walls lost. Inter-examiner agreement for articles inclusion resulted in a Cohen's *k*-score of 0.90. The study selection process is depicted in figure 1. Characteristics of the included studies are described in table1.

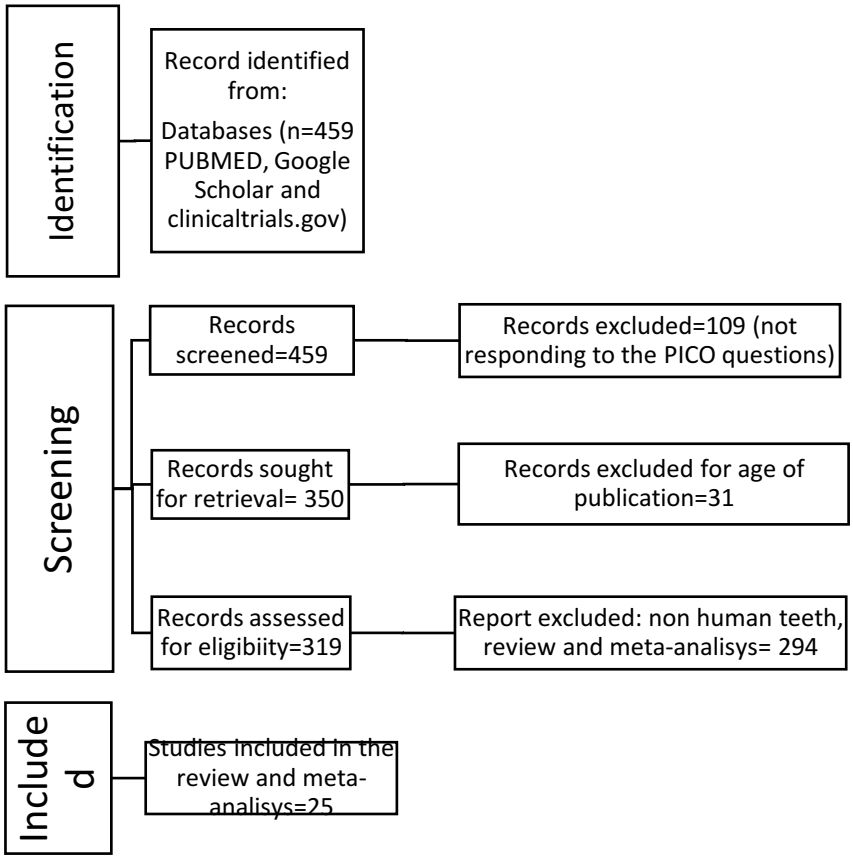


Figure 1: PRISMA flow diagram summarizing all inclusion criteria.

Authors	Year	Teeth	N	PDL	Thermocycling	Load inclination	Tip application
Göktürk	2018	Upper	55	+	+	90°	Buccal / Lingual cusp
Oz	2019	Upper	80	+	+	90°	Occlusal surface
Eapen	2017	Upper	60	-	-	90°	Occlusal inclines of the Buccal and Lingual cusps
Harsha	2017	Upper 1st	40	-	-	30°	On the Center of the Buccal cusp
Desai	2011	Upper 1st	30	-	+	90°	Center occlusal surface
Kemaloglu	2015	Lower	48	+	+	90°	B and L cusps simultaneously
Zogheib	2018	Upper	60	+	-	90°	Central fossa
Karzoun	2015	Upper	60	+	-	90°	/
Taha	2014	Upper	48	-	-	45°	/
Hshad	2018	Lower	48	-	-	90°	Interdental surface of buccal cusp
Angol	2013	Upper	10	-	-	90°	Simultaneous contact B and P cuspal inclines
Taha	2011	Upper	80	-	-	45°	Palatal incline of the Buccal cusp
Taha	2015	Upper	77	-	-	45°	Palatal incline of the Buccal cusp
Mashyakhy	2020	/	52	-	+	90°	Center occlusal surface
Spicciarelli	2020	Upper 1st (1 root)	165	-	-	90°	2mm from apex of Palatal cusp in the direction of Central fossa
Monga	2009	Upper	80	-	-	90°	Occlusal inclines of the buccal and lingual cusps
Soares	2008	/	50	+	-	45°	Buccal and Lingual cusp
Sengun	2008	Lower	80	+	-	45°	Central fossa with Lingual orientation
Soares	2008	Upper (1 root)	60	+	-	90°	Center occlusal surface
Shahrbaf	2007	Upper	90	-	+	45°	Palatal cusp
Coelho	2005	Upper	40	-	-	90°	Buccal and Lingual inclined cuspal planes, not restoration
Mondelli	2007	Upper 1st	30	-	-	90°	Contacted both cusps simultaneously
Skupien	2016	Upper (1 root)	20	-	+	45°	Toword buccal cusp
Plotino	2017	Upper 1st	80	-	-	30°	Central fossa
Gürel	2016	Upper (1 root)	48	-	-	30°	Central fissure of the occlusal surface

Table 1: characteristics of the included studies.

3.2 Descriptive analysis

Intact premolars

Out of 17 subgroups, the load inclination applied was 90° for nine[10-13,16,19,25,28,31] and 30°/45° for eight[33,20-21,24,30-31]. PDL simulation was performed in five subgroups[10,11,16,24] while thermocycling was applied in five[10,11,16,30]. Tip diameter was respectively lower than 2mm and between 2 and 3 mm in one group, between 3 and 5 mm in four groups, 6mm in seven groups and 8mm in two groups.

Premolar with 0 wall lost

Out of 17 subgroups, the load inclination applied was 90° for two[15] and 45° for six[23,25,31]. Thermocycling simulation was performed in two subgroups[25] while no PDL simulation was applied. Tip diameter was respectively 3 mm in three groups and 6 in seven.

Premolar with 1 wall lost

Out of 6 subgroups, the load inclination applied was 90° for four[13,23,27] and 45° for two[13]. Thermocycling was performed in one subgroup[13] while no PDL simulation was applied. Tip diameter used was between 2 and 3 mm in one group while in the remaining was not specified.

Premolar with 2 walls lost

Out of 45 subgroups, the load inclination applied was 90° for twenty-four[10,11,14,24,27] and 30°/45°for twenty-one[10,12,16,23,29-30]. PDL simulation was performed in twelve subgroups[10,14,16,24,27] while thermocycling was applied in six[10,14,27]. Tip diameter was respectively 3 mm in three groups and 6 in seven. Tip diameter was respectively lower than 2mm in nine and between 2 and 3 mm in one group: between 3 and 5 mm

in four groups, 6mm in thirteen groups and 8mm in seven. In remaining groups, the tip diameter was not specified.

3.3 Meta-analysis

Intact premolars

Thirteen groups were included for meta-analysis with very high heterogeneity value (I^2= 94.98%) (Fig.2) while publication bias was not present (p>0.05). Subgroup analysis showed that the presence of PDL simulation did not influence the fracture strength value in intact premolars (p>0.05) while 90° load inclination affect significantly the results respect to 30/45° (p=0.00) (Tab. 2). Meta-regression of the tip diameter did not influence the fracture strength of intact premolar (Tab. 5).

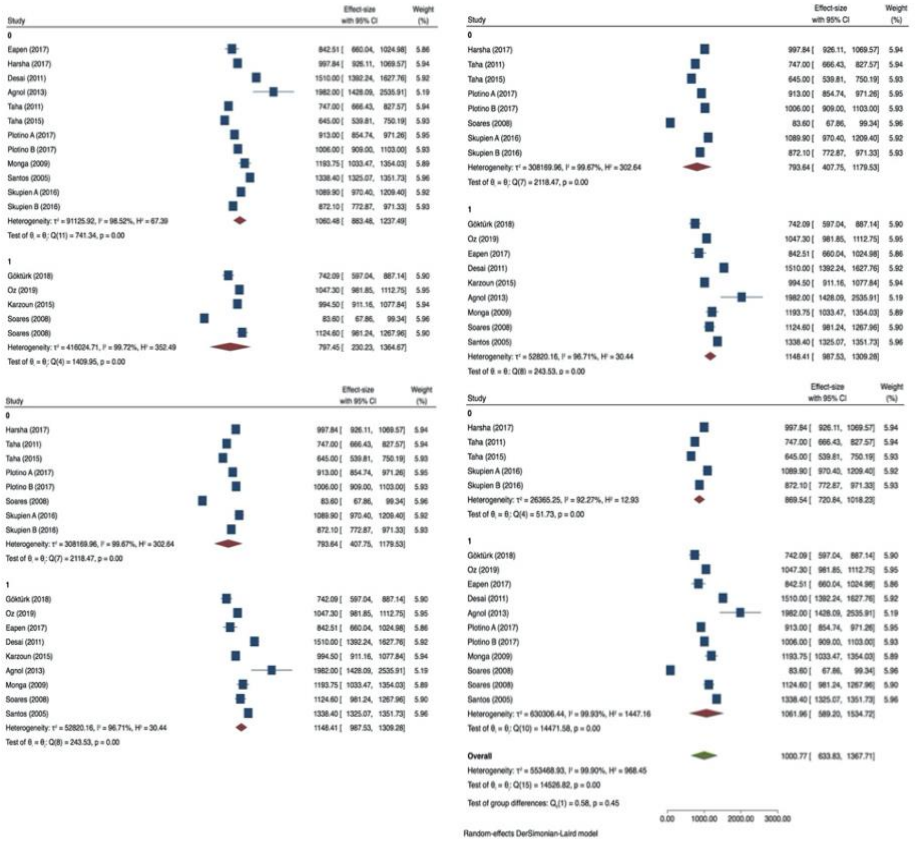


Fig. 2: Forest plots for intact premolars of PDL simulation, Thermocycling, Load inclination and Tip position.

Heterogeneity summary: **PDL simulation** (0= - PDL; 1= +PDL)						Number of studies: 17
Group	df	Q	P > Q	Tau²	%I²	H²
0	11	741,34	0,000	91125,915	98,52	67,39
1	4	1409,95	0,000	4,16e +05	99,72	352,49
Overall	16	14540,83	0,000	5,38e +05	99,98	908,80
Test of group differences: Q__b = chi²(1)= 0,75						Prob > Q__b = 0,386
Heterogeneity summary: **Load Inclination** (Binary; 0= 30/45°; 1= 90°)						Number of studies: 17
Group	df	Q	P > Q	Tau²	%I²	H²
0	7	2118,47	0,000	3,08e +05	99,67	302,64
1	8	243,53	0,000	52820,161	96,71	30,44
Overall	16	14540,83	0,000	5,38e +05	99,98	908,80
Test of group differences: Q__b = chi²(1)= 2,77						Prob > Q__b = 0,096
Heterogeneity summary: **Tip Position** (0= either buccal or lingual; 1= central fossa or both cusps)						Number of studies: 17
Group	df	Q	P > Q	Tau²	%I²	H²
0	4	51,73	0,000	26365,250	92,27	12,93
1	10	14471,58	0,000	6,30e +05	99,93	1447,16
Overall	15	14526,82	0,000	5,53e +05	99,90	968,45
Test of group differences: Q__b = chi²(1)= 0,58						Prob > Q__b = 0,447
Heterogeneity summary: **Thermocyling** (0= -Therm.; 1= +Therm.)						Number of studies: 17
Group	df	Q	P > Q	Tau²	%I²	H²
0	11	14351,13	0,000	5,93e +05	99,92	1304,65
1	4	88,66	0,000	58364,924	95,49	22,16
Overall	16	14540,83	0,000	5,38e +05	99,98	908,80
Test of group differences: Q__b = chi²(1)= 0,09						Prob > Q__b = 0,766

Table 2:heterogeneity summary, PDL Simulation, Load Inclination, Tip Position, Thermocycling in intact premolar subgroup.

Premolar with 0 wall lost

A Meta-analysis was performed with 10 groups. The Heterogeneity was very high (I^2= 97.73%) (Fig.3), but in this case the publication bias was absent (p=0.00). PDL simulation significantly influences the fracture strength test (p=0.00), as well as the 90°tip inclination when compared with 30/45°(p=0.00) (Tab. 3). Tip position and thermocycling doesn't affect the fracture strength test of premolars with 0 wall lost (p>0.05). Meta-regression of tip diameter does not seem to affect the test (p>0.05).

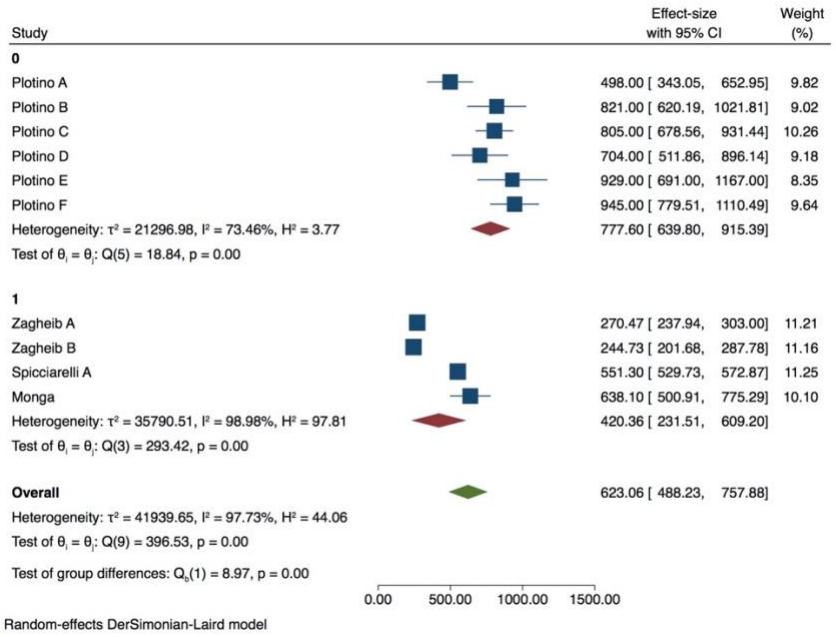


Fig. 3: Forest Plot of Premolar with 0 wall loss of Tip inclination variable.

Heterogeneity summary: **Load Inclination** (Binary; 0= 30/45°; 1= 90°) Number of studies: 10

Group	df	Q	P > Q	Tau²	%I²	H²
0	5	18,84	0,002	21296,981	73,46	3,77
1	3	293,42	0,000	35790,506	98,98	97,81
Overall	9	396,53	0,000	41939,649	97,73	44,06

Test of group differences: Q__b = chi²(1)= 8,97 Prob > Q__b = 0,003

Table 3: Summarizing of results in subgroups with 0 walls lost

Premolar with 1 wall lost

6 groups were included in the meta-analysis. Despite a high heterogeneity (I^2= 97.73%) (Fig.4), a publication bias was present (p>0.05). Tip position and thermocycling significantly influence the test on premolar with 1 wall lost (p=0.00), while inclination did not (p>0.05) (Tab. 4).

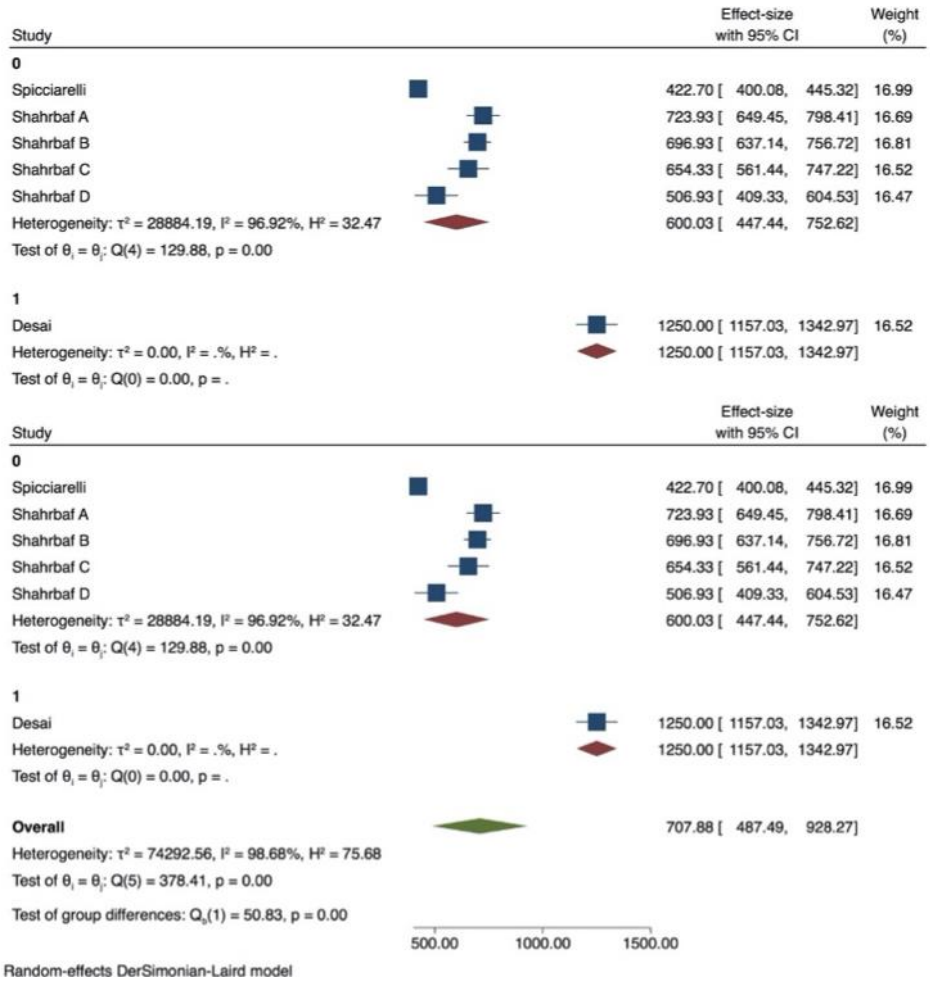


Fig. 1: Forest Plots of Thermocycling and Tip Position in premolar with 1 wall lost.

Heterogeneity summary: **Load Inclination** (Binary; 0= 30/45°; 1= 90°)						Number of studies: 6
Group	df	Q	P > Q	Tau²	%I²	H²
0	0	0,00	.	0,000	.	.
1	4	378,35	0,000	84712,462	98,94	94,59
Overall	5	378,41	0,000	74292,555	98,68	75,68
Test of group differences: Q__b = chi² (1)= 2,94						Prob > Q__b = 0,086

Heterogeneity summary: **Thermocyling** (0= -Therm.; 1= +Therm.)						Number of studies: 6
Group	df	Q	P > Q	Tau²	%I²	H²
0	4	129,88	0,000	28884,187	96,92	32,47
1	0	0,00	.	0,000	.	.
Overall	5	378,41	0,000	74292,555	98,68	75,68
Test of group differences: Q__b = chi² (1)= 50,83						Prob > Q__b = 0,000

Tab. 4: Summarizing of load inclination and thermocycling subgroup results with 1 wall lost

Premolar with 2 walls lost

A meta-analysis conducted with 45 groups showed a very high heterogeneity (I^2= 97.73%) (Fig.5), and the Egger test didn't indicate publication bias (p<0.05). PDL simulation showed no influence on the fracture strength test (p>0.05). 90° tip inclination significally affected the test compared to 30/45°(p=0.05). Neither the tip position nor the diameter seemed to influence the results of the in vitro test (p>0.05).

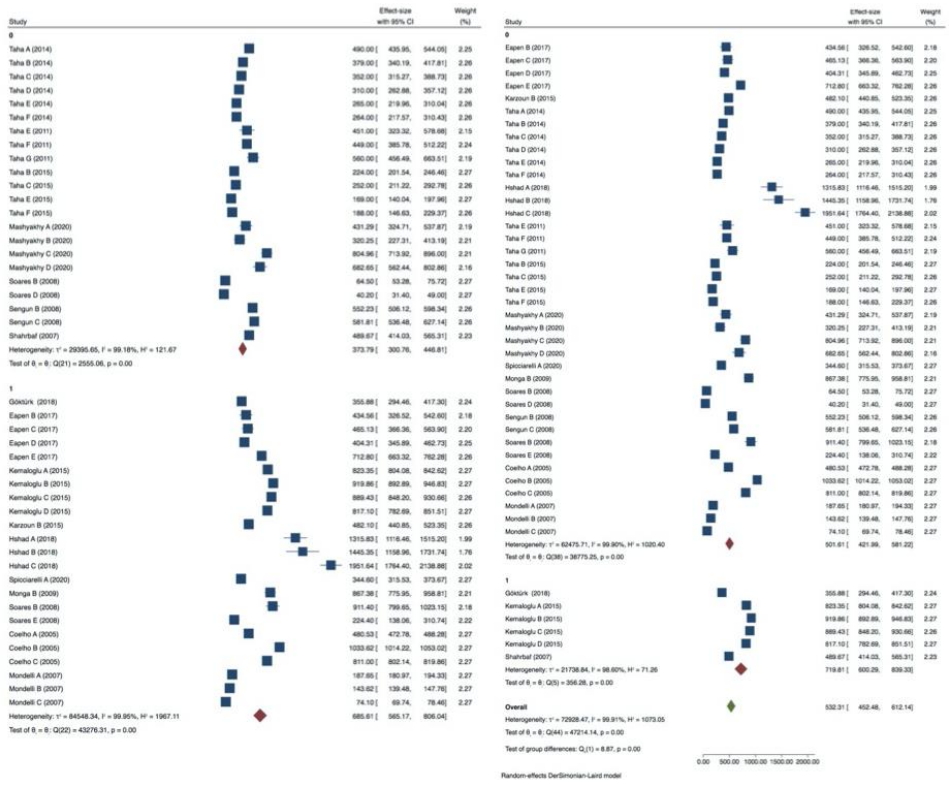


Figure 5: Forest Plot of Tip inclination and Thermocycling in Premolar with 2 walls lost.

_meta_es	Coef.	Std. Err.	z	P > |z|	[95% Conf. Interval]	
Tip diameter	108,9722	48,74386	2,24	0,025	13,43596	204,5084
_cons	92,22167	251,3299	0,37	0,714	-400,3759	584,8193

Test of group differences: Q_res = chi^2(13) = 396,23 Prob > Q_res = 0,0000

_meta_es	Coef.	Std. Err.	z	P > |z|	[95% Conf. Interval]	
Tip diameter	54,2533	21,42337	2,53	0,011	12,26426	96,24234
_cons	272,2454	114,956	2,37	0,018	46,93586	497,555

Test of group differences: Q_res = chi^2(13) = 18037,89 Prob > Q_res = 0,0000

Table 5: Meta-regression of tip diameter in subgroup with 0 and 2 walls lost

4 Discussion

Summary of findings

In the present meta-analysis, we evaluated the impact of methodological variables such as periodontal ligament simulation, tip diameter, position and inclination, thermo cycling in the fracture strength test on the upper premolar with different

residual walls. Studies regarding premolars were chosen because of their natural tendency to be more prone to fracture compared to other teeth, although anatomical variables such as crown volume and inclination of the cusp could affect the strength of the tooth. According to the results of the present study, all methodological variables, including periodontal ligament simulation, thermocycling, tip position and diameter, load inclination don't seem to affect fracture strength test in intact premolar, whereas a very significant publication bias and high heterogeneity was present in this subgroup. In this group the fracture strength does not seem to be affected by the variables analyzed, showing, as expected, the higher value of fracture strength compared to other groups as confirmed previously by da Angol et al.[19] and Jantarat et al.[35]. This could be explained by the homogeneous distribution of load force transferred from the rigid intact enamel to the underlying dentin as demonstrated by Ausiello[36]. Periodontal ligament simulation influences the fracture strength test only in premolar with 0 wall lost respect the other three groups examined in this review. Periodontal ligament represents an important anatomical structure able to absorbe the occlusal forces during chewing function and should be reproduced in the "in vitro test" in order to simulate the clinical reality. Rees underlined the importance of simulating the periodontal ligament in the fracture strength test by a finite element analysis Anyway, many materials were utilized to perform these procedures and a lack of uniformity between studies was observed in the review analysis.

Metaregression regarding the variable tip diameter seemed to not influence the test in any case. Also in this case new studies needed to understand if the diameter of the tip could influence the fracture strength test. The load inclination seemed to influence the fracture strength test when the force was applied at 30/45° instead that perpendicular, except for premolars with 0 wall lost. Yang[37] showed that the direction of the force decreases the fracture strength as the walls lost increase. The

same results were obtained by Reeh et al.[4] in case of endodontically treated teeth, demonstrating a higher resistance to fracture when conservative cavity access was applied. According to our data, thermocycling seems to influence with statistical significance the data of fracture strength test mostly in case of premolars with 1 and 2 walls lost, showing higher resistance to fracture when the procedure was applied. Our results are in agreement with Sabery regarding intact premolars whereas his showed that Thermocycling influenced the fracture strength values in case of different cavity preparation. This data could be explained by the fact that thermocycling is applied to put in evidence teeth with crack or fracture already present which could influence the results of the test.

Strengths and limitations

The current review represents the first meta-analytical analysis of the methodological variables present in the fracture strength and their influence on the test results. The strict methodology used according with new PRISMA guidelines, the high number of the included study as well as the high number of the methodological variables analyzed represent the strength of the present study. Despite the lack of publication bias, outcome reporting bias could be present, affecting the heterogeneity of the selected studies. In fact, despite additional analysis, it was impossible to reduce the heterogeneity, thus suggesting that other methodological variables, as well as ignoring non-significant outcomes, which could not be considered, may have influenced the results. The present meta-analysis showed un high heterogeneity values in all groups analyzed and represent the main limitation of the present study in which the results should be considered with caution. Data were scarce about periodontal ligament simulation, tip position and diameter and in any case, our meta-analysis fails to detect a statistical significance in the

fracture strength test, despite it has been observed that a decrease of the tip diameter matches a lower resistance to fracture[38]. Another limitation of this systematic review is represented by the lack of information regarding the choice of what upper premolar has been used. Taha et al[5] reported the influence of shape difference of cervical area between first and second premolar in the fracture susceptibility.

5 Conclusion

Fracture strength test is the main in vitro study able to better understand the capacity of dental materials to resist under stress conditions and in various clinical situations. Despite the numerous studies already published in the literature, there is an evident lack of uniformity. The present meta-analysis highlights the necessity to standardize the procedure in order to reduce the variability of fracture strength test results.

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4. Comparison of physico-chemical properties of new zinc oxide eugenol cement and a new bioceramic sealer.

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Aust Endod J. 2022 Nov 21.

Introduction

The long-term success of root canal treatments is mainly due to the chemo-mechanical disinfection of root canal system, and the stability of both coronal and apical obturation [1, 2]. The success is provided by root canal chemo-mechanical debridement aiming to reduce bacterial load [3], although shaping and irrigation procedures are not able to completely remove bacteria from the endodontic system [4]. The persistence of microorganisms in the root canal system remains the main cause of endodontic failure [5]. For this reason, the obturation procedures are essential to obtain the bacterial entombing to prevent their proliferation. Moreover, Gutta-percha, in conjunction with sealer, could play a key role in the sealing ability of the root apex, preventing interstitial fluid permeability from the periapical tissue [6,7]. One of the most common causes of failure is represented by inadequate quality sealing of root canal system [8]. According to this, the choice of the obturation materials, both in terms of physico-chemical and biological aspects, is crucial to determine root canal treatment success rate. Root filling materials need to meet several requirements in terms of radiopacity, flowability, biocompatibility and setting time [9]. According to this, a correct selection of the most suitable endodontic sealer, requires a deep knowledge of its features. An ideal endodontic sealer has to respect specific physico-chemical properties such as the ability to provide an hermetic microscopic seal, good

adhesion to dentin, dimensional stability, sufficient setting time, tolerance to fluids and biocompatibility. Anatomical accommodation provided by the presence of fine powders, radiopacity, color stability, bacteriostatic and antibacterial properties are other compulsory requisites. On the contrary, it should not be mutagenic, sensitizing and cytotoxic after setting [10]. At present, various products are commonly utilized in clinical practice, including sealer based on resin, zinc oxide-eugenol, calcium hydroxide, glass ionomer and bioceramics. Among the most widely used endodontic sealers, zinc oxide eugenol sealers have a long tradition in both clinical practice and scientific research [11]. Calcium silicate-based sealers, precisely named as bioceramic sealers, were introduced into the market as a possible substitute to traditional endodontic sealers [12]. Recently a new Zinc oxide-eugenol cement was introduced into the market as an alternative to both new and past root canal sealers. In order to assess the quality of a recently introduced sealer, the purpose of the present in vitro study was to evaluate flow properties, setting time, radiopacity, solubility, film thickness of new endodontic sealer EssenSeal (ES) (Produits Dentaires SA, Vevey, Switzerland), when compared to AH Plus Bioceramics (AH) (Dentsply International, TN, USA) and Pulp canal sealer EWT (PCS) sealers (Kerr Corporation, Orange, CA, USA). Furthermore, the morphologies of the external surface and the cross-section of all sealers were also quantitatively assessed under scanning electron microscopy (SEM). The null Hypothesis was that the tested sealers have comparable characteristics in terms of flow properties, setting time, radiopacity, solubility, film thickness and microleakage.

Materials and Methods

EssenSeal, Pulp Canal Sealer EWT and AH Plus bioceramic were tested for flow, setting time, solubility, radiopacity and film thickness according to ISO 6876 specification [13]. The properties and characteristics of the sealers tested are shown in table 1. All materials were prepared and used according to manufacturer' instructions. EssenSeal was prepared by mixing one level dosing spoon of powder and one drop of liquid and gradually incorporating

the powder into the liquid, until a creamy consistency was obtained, fluid enough to stretch out for 2 cm on the mixing block. Pulp Canal Sealer EWT was prepared by mixing one level dosing spoon of powder with one drop of liquid and incorporating the powder into the liquid, mixing them in a very small area of about 1-2 cm diameter with a stainless-steel spatula. AH Plus bioceramic is a single paste, therefore the mode of use consists only in inserting the 24-gouge tip no farther than the middle third of the root canal. For each sample undergoing test, 0.3 g of sealer was distributed on a mixing pad. With the intention to standardize and blend the material, the sealer was mixed with a stainless-steel spatula for 15 s.

Flow test

ISO 6876 international standard version 2012 was used to conduct the flow test which requires that a sealer shall have a diameter of at least 17 mm. 0.05 mL of the material was mixed and placed on the center of a glass plate, with a weight of 20 g and dimensions of 20 mm, using a graduated syringe (Becton Dickinson, Curitiba, PR, Brazil). At 180 s (±5 s) after beginning the mixing, another glass plate was placed centrally on top of the sealer, followed by a weight giving a total mass of 120 g (±2 g). Ten minutes after initiating the mixing, the weight was removed and the maximum and minimum diameters of the compressed discs of sealers were measured. Two conditions were necessary to validate the tests: the difference between the maximum and minimum diameters could not exceed 1.0 mm and the compressed disc should have uniform shape. If these conditions were not met, the test was repeated. Three determinations were carried out and the mean value was calculated to the nearest millimeter. The disc diameter should be at least 17 mm.

Solubility

Solubility test was conducted using the ISO 6876 (2012) standards, according to which the sealer solubility shall not exceed 3.0 % by mass. Cylindrical polytetrafluoroethylene molds with an inner diameter of 7.75mm and a height of 1.5mm were respectively filled with each type of extemporaneously mixed sealer with the adjunct of a standardized waterproof nylon thread, applied to

facilitate the handling procedures. Each sample was incubated at 37 °C and 95% relative humidity for 24 h and then removed from the mold and weighted three times each (m1) with an accuracy of 0.0001 g (Gibertini 500; Gibertini elettronica SRL, Novate, Milano, Italy). After that, samples were suspended in a plastic container of 7.5 mL milli-Q by the use of the nylon threads to avoid any contact between sample and container. Each container was placed in the incubator at 37 °C and 95% relative humidity. After 7 days, the samples were removed from the incubator, rinsed with deionized water and dried at 60 °C. Afterwards, each sample was weighed again (m2). The solubility was measured by calculating the weight loss of each sample (m1-m2) and expressing it as percentage of the original mass, using the following formula: (m1-m2)/m1*100%.

Setting time

Setting time was also tested using the ISO 6876 (2012) standards stating that a sealer shall be no more than 10% respect the declared value by the manufacturer. Five Plaster of Paris cast rings of 10 mm diameter and 2 mm thickness were separately filled with one of the selected sealers and then inserted in an incubator at 37 °C and 95% relative humidity for 2 min. Afterwards, a penetrometer (NEWTRY GY-3, Huizhou, Cantón, China) with a weight of 100 g and a diameter of 2mm was used to evaluate the samples hardness, by carefully lowering its flat tip in a perpendicular direction against sealer discs. The setting time was considered as the difference in terms of seconds between the end of sealer mixing and the moment at which indentations were clearly visible. 3 measurements were performed for each sealer.

Radiopacity

A sealer shall have a radiopacity less than 3 mm of aluminum in accordance with the ISO 6876 (2012) standards implemented to test radiopacity. Plaster of Paris cast rings of 5 mm diameter and 1 mm thickness were separately filled with one of the selected sealers and then incubated at 37 °C and 95% relative humidity for 24h. The filling procedures were performed by the use of a syringe in order to avoid bubbles formation.

Each sample was positioned alongside an aluminum wedge with the thickness varying from 0.5-6 mm in order to uniform steps of 1 mm each and three digital radiographs per each sealer were taken (KAVO, Dental Imaging Technologies Corporation, 1910 North Penn Road Hatfield, PA, USA) at 60 kV and 2.5 mA with focus-film distance of 30 cm and exposure time set to 0.16 s, maintaining the aluminum wedge as an occlusal contrast standard reference. Grey pixel values of each sealer and aluminum wedge on the images were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The radiopacity of the samples was expressed in millimeters of aluminum (mm Al) by comparing the grey values of sealers and the calibrated aluminum wedge, according to the plotting with the best-fit trend line of the graph of the thickness of aluminum, against grey pixel values.

Film thickness

Film thickness was assessed using the ISO 6876 (2012) which requires a film thickness lower than 50um. Each mixed sealer was placed between two glass plates characterized by a thickness of 5 mm and a surface of 200 ± 25mm^2. Before sealer placement the thickness of the two glass plates was measured

with a caliper (Dongguan Kuaijie Measuring Tool Instrument Co., Ltd., Dongguan, China) to obtain the reference thickness starting point. After 180 ± 10 s from the start of mixing, a load of 150 N was vertically positioned for 10 minutes onto the glass plate, allowing the flow of the sealer on the entire area between the two glass plates. Afterward, the thickness of the two glass plates with the sealer layer was measured (final thickness), and the difference in terms of mm between the reference thickness starting point and the final thickness was determined as film thickness. Three measurements were performed for each sealer.

SEM examination

In order to perform SEM examination, freshly mixed sealers were poured in cylindrical polytetrafluoroethylene molds. A glass plate covered with a cellophane sheet was used to sustain the molds and placed in a chamber (37 °C, 95% RH) for a period equivalent to three times the setting time. Through the use of a size 15 disposable surgical scalpel blade, fixed on a metallic stub (10 x 5 mm), samples were consequently sectioned and spatter coated with gold-palladium (Bal-Tec AG, Balzers, Germany) at 20 mA. Quantitative analysis of the sample external surface characteristics and cross section was achieved under a field emission SEM (GEOL JSM-6060, Westmont, USA). The examination was carried out at diverse magnifications, at a working distance from 6 to 10mm and at an accelerating voltage of 15 kV.

Statystical analysis

Normality of data distribution was checked both visually and using the Shapiro-Wilk test. After verification of data distribution, a one-way ANOVA test was used to compare all the study groups; Tukey's HSD test was used for pairwise comparisons. The level of significance was set at alpha=0.05; all p-values were two-sided.

Results

Flow test

The ISO 6876 requirement was satisfied by all the tested sealers. PCS group showed a greater flow (Mean= 30.17, SD ± 2.17) followed by ES (Mean= 27.64, SD ± 0.85) and AH (Mean= 25.46, SD ± 0.99). Statistically significant differences were present when AH was compared to PCS group (P=0.000), whereas no difference was observed between ES and PCS groups (P>0.05) (Table 2).

Solubility

The sealers tested were respectful of the standards required. The ES group showed a higher value of solubility (Mean= 0.144, SD ± 0.003) followed by AH (Mean= 0.137, SD ± 0.006) and PCS (Mean= 0.120, SD ± 0.003). Statistically significant differences were present when ES were compared to AH group (P<0.001) (Table 2).

Setting time

AH group had a stated setting time between 2 (180') and 4 (240') hours, thus did not meet the standards indicated by the manufacturer (Mean= 408, SD ± 15.37). ES (Mean= 119.80, SD ± 0.78) and PCS groups (Mean= 590, SD ± 7.90) respected the setting time established respectively in 2 to 4 and more than 6 hours. Statistically significant differences were present between all groups tested (P=0.000) (Table 2).

Radiopacity

All groups showed radiopacity values above that requested by ISO 6876 standard. Similar values were observed between AH (Mean= 9.83, SD ± 1.28) and ES (Mean= 9.52, SD ± 0.89) groups whereas PCS group showed a lower radiopacity (Mean= 5.92, SD ± 2.49). Statistically significant differences were present when PCS were compared to both AH (P<0.001) and ES group (P<0.05) (Table 2).

Film thickness

All sealers satisfied the standard although a statistically significant difference was present between groups (P=0.000). PCS group showed a lower film thickness (Mean= 9.8, SD ± 0.11) followed by AH (Mean= 10.24, SD ± 0.11) and ES (Mean= 10.78, SD ± 0.13). Statistically significant differences were present when AH were compared both to PCS (P=0.000) and ES group (P=0.000) whereas no difference was observed between ES and PCS groups (P>0.05) (Table 2).

SEM examination

Scanning electron microscopy revealed sphere-shaped polymers of different size homogeneously spread on the external surface in both PCS and ES samples. ES polymers appear to be more voluminous than PCS ones. In the PCS sample, a more uniform and organized layer with a higher number of polymers in a reduced resin matrix was appreciated, in contrast to ES. AH Plus bioceramic samples revealed a regular surface with homogenous, smaller and globular-like particles of different sizes (smaller than ES and PCS groups). The material matrix was dense, and the phases were uniformly distributed. At 2000x magnification shrinkage lines can be noted, probably arising from cutting procedures (Fig. 1).

Discussion

The aim of the present study was to evaluate flow, solubility, radiopacity, film thickness and time setting properties of a new Zinc oxide-eugenol based sealer EssenSeal compared to both Pulp canal sealer and AH plus bioceramics according to ISO 6876 standards. The results of this study showed that all sealers tested differs for the physicochemical properties analyzed. Thus, the null hypothesis was rejected. All the sealer tested satisfied the ISO 6876 standard for the physicochemical properties analyzed except for setting time of AH Plus bioceramic that exceeded more than 10% the time indicated by the manufacturer. This could be partly explained by the fact that calcium-

silicate based cement needs moisture present in the dentinal walls to improve the setting reaction [14]. Recently, Almeida et Al showed similar or, in some cases, better results about physicochemical properties of bioceramic compared to traditional sealers [15-16], consequently in vivo and ex vivo studies are more accurate in the evaluation of setting time of this type of sealer [17]. Whereas with small differences between groups, EssenSeal showed a higher value in solubility with respect both to AH and PCS groups. According to our result, AH group respected the standards and disagreed with recent study in which a calcium-silicate based sealer did not satisfy the ISO 6876 requirements [18]. Flow property plays a crucial role because it allows proper sealer penetration into the endodontic system. According to the standard requirements, the sealer during flow test should have a diameter of no less than 17mm with a standard deviation of 1mm. All the sealers tested respected the minimum demanded flow value, PCS group showed the highest results respect to ES and AH. The flowability results of PCS of this research are slightly different in comparison to those of Donnermeyer [19]. Regarding this, the authors tested the physico-chemical properties of different endodontics sealers (PCS included) at different temperatures, stating that there were no differences in terms of physico-chemical properties when heating did not exceed 60s. Despite this, considering their PCS results obtained at 20 °C, they found lower flowability values than that obtained in this study. The flow characteristics seem to be influenced by particle size in the powder part of the sealers, as well as the setting time. In 1982, Grossman [20] showed the better plasticity of zinc eugenol resin-based and demonstrated improvement of flow test of this sealer, despite the flow values

are sensibly inferior to that obtained by Siqueira [21]. In any case, rate of shear, time setting and temperature could sensibly affect the flow value justifying, in part, the different values obtained. Radiopacity represents a physical property that allows the clinician to better evaluate radiographically the sealing quality of endodontic treatment. Despite the constitution of AH plus bioceramic presents some radiopacifier, such as zirconium oxide and calcium tungstate, ES group showed the same value in terms of radiopacity determining for both a significant difference when compared to PCS group. The calcium-silicate sealer used in this study seems to have better radiopacity properties in comparison with the one tested in a recent study [22]. This could be explained by the fact that presence, absence and amounts of some radiopacifier agents might produce a more or less radiopaque cement [23]. The PCS group had lower value whereas previous study reported higher value in terms of radiopacity [24]. Silver is the principal radiopacifier in zinc-oxide eugenol-based sealers and thus the variation of powder/liquid ratio could sensibly modify the amounts of these agents affecting the radiopacity value. The film thickness standards formulated by ISO 6876 requirements were widely satisfied from all the sealers tested, showing greater values for AH and ES with respect to PCS groups. For several years epoxy-resin and zinc oxide-eugenol sealers have been considered the gold standard in endodontics. Recently calcium-silicate based endodontic sealers were proposed as valid alternative to the traditional widely used sealers. Even though, all the sealers tested confirmed the data of the manufacturer, respecting the minimum standards required. This in vitro study does not take into account some variables related to the real clinical environment that could alter the physico-

chemical performance of the tested sealer. There are some questions indeed about the actual use of the sealers in specific clinical procedures that should be addressed with further research. Due to the lack of reliable data on clinical outcomes, ex vivo and in vivo studies are required to better evaluate the real performance in the long-term clinical outcomes. However, in vitro studies are the best predictable and repeatable way to evaluate sealer properties, considering that results of in vivo studies could be altered by uncontrolled factors. In accordance with this, in vitro studies can be considered as the first level of evidence, essential for an initial evaluation, that should be implemented with ex vivo and in vivo studies.

Conclusion

All tested sealers meet the minimum values required by ISO 6876 standards except for setting time in the case of AH plus bioceramics. As a rule, a fast-setting time renders technical difficulty during the application whereas a slow setting time or an incomplete set can result in higher tissue irritation and increasing solubility, possibly leading to sealing failure. Besides the limitations of the present in vitro study, EssenSeal satisfied all the ISO 6876 (2012) standard requirements. Given its excellent characteristics of flowability, radiopacity, film thickness and setting time, it can be considered a promising alternative to the sealers already on the market.

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Table 1. Characteristics of sealers employed.

Product name (Manufacturer, city, country)	Chemical matrix	Component	Composition
AH PLUS (Dentsply DeTrey, Konstanz, BadenWürttemberg, Germany) *	tricalcium silicate	Single paste	Zirconium Dioxide, Dimethyl sulfie, Lithium carbonate, Thickening agent
PULP CANAL SEALER EWT (Kerr, Brea, CA, USA)	Zinc oxide-eugenol	Powder	Zinc oxide, precipitated silver, oleo resin, thymol iodide
		Liquid	Oil of cloves, canada balsem
ESSENSEAL (Produits dentaires, SA, Vevy, Switzerland)	Zinc oxide-eugenol	Powder	Zinc oxide, barium sulphate, excipient
		Liquid	Eugenol, tea tree essential oil (Maleleuca), excipient.

Table 2: Physicochemical properties of sealers tested.

	Sealers tested		
	I	II	III
	AH	PCS	ES
Setting time (s) ***	**408 ± 15.37^{B}**	590 ± 7.90^{ac}	119.8 ± 0.78^{ab}
Radiopacity (mm) **	9.83±1.28	5.92±2.49^{a}	9.52±0.89^{b}
Flow (mm) **	25.46 ± 0.99	30.10 ± 2.17^{a}	27.64 ± 0.85
Solubility (g) **	0.137 ± 0.006	0.120 ± 0.003	0.114 ± 0.003^{a}
Film Thickness (µm) ***	10.24±0.11^{b}	9.8±0.07^{a}	10.78±0.13^{ab}

Values are mean ±standard deviation. Values in bold letters do not comply with ISO 6876 standard. Values followed by different superscript letters in each row differ significantly. One way Anova test between groups: **p<0.001, *p=0.000.

5

5. Antimicrobial peptides active against periodontal bacterial infections modulate inflammation in human cardiac fibroblasts

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INTRODUCTION

Antimicrobial resistance is a major threat to human health. It is responsible for an estimated 33,000 deaths per year in the EU [1-2] and comes with a heavy economic burden. The lack of effective new antimicrobials is due to little investment in antibiotic research and to excessive use and misuse of antibiotics to treat humans and animals. Trivial and life-threatening infections are often treated with the same antibiotics, generating an increasing number of resistant strains that can easily turn a trivial bacterial infection into a severe disease.

Endodontic and periodontal disease begin by bacterial colonization. Though initially local, chronic infection may become a source of antimicrobial-resistant bacteria. Chronic disease also triggers sustained release of pro-inflammatory mediators that play an essential role in the progression of inflammation [3-4] and can eventually have detrimental effects on distant organs. Many epidemiological studies suggest that periodontitis is associated with atherosclerotic cardiovascular disease [5-7]. Bacteria enter the systemic

circulation, stimulating atherogenesis through endothelial damage and inflammation, as shown by the fact that experimental bacteraemia induced by *P. gingivalis* in animals leads to atherogenesis [8]. Furthermore, patients with periodontitis constantly produce proinflammatory cytokines that can reach the liver and trigger release of acute-phase response proteins (alpha 1-acid glycoprotein, C-reactive protein, fibrinogen and serum amyloid A), causing endothelial damage and triggering atherogenesis [9].

The human oral microbiota is known to contain as many as 700 microorganisms. The average person has about 250 species. They include Gram-negative bacteria, such as *Escherichia coli*, *Porphyromonas gingivalis* and *Pseudomonas aeruginosa*, Gram-positive bacteria, such as *Actinomycetes, Enterococci, Lactobacilli, Staphylococci* and *Streptococci,* with *Streptococcus mutans*, which causes dental caries, and fungi, mainly *Candida*. Among the microorganisms causing periodontitis, Enterococci are major players [10].

Enterococci are Gram-positive colonizers of the mouth and mammalian gastrointestinal tract and normally live in healthy association as commensals in humans and animals [11]. However, *Enterococcus faecalis* can cause bacteraemia and severe infections such as meningitis and endocarditis [12]. In the oral cavity, *E. faecalis* has been detected predominantly in the root canals of patients with post-treatment apical endodontitis or refractory apical periodontitis, suggesting an etiological role in progression of these diseases [13-14]. The emergence of antibiotic-resistant *E. faecalis*, such as vancomycin-resistant *E. faecalis* (a glycopeptide-resistant strain) prompted research and development of new antibiotics.

In dentistry, antimicrobial peptides have been suggested to fight pathogens in implants and dental adhesives [15]. The peptide M33D is a tetra-branched peptide active against Gram-negative and Gram-positive bacteria [16-17]. It has long-life, unusual for peptides, since the branched form imparts resistance to plasma, serum and bacteria proteases [18].

M33D shows MICs of 0.7-6.0 uM (4-32 ug/ml) against multiresistant pathogens of clinical interest, such as Gram-positive *Staphylococcus aureus, Staphylococcus saprophyticus* and *Enterococcus faecalis,* including a

vancomycin-resistant strain, and various Gram-negative enterobacteriaceae [17]. The activity is accompanied by a low frequency of resistance selection [17].

M33D has been successfully tested against multi-drug resistant strains of seriously dangerous Gram-negative species, such as *Escherichia coli*, *Klebsiella pneumonia, Acinetobacter baumannii* and *Pseudomonas aureoginosa,* resistant to carbapenem, extended-spectrum cephalosporin, fluoroquinolone, aminoglycoside and importantly, colistin [17].

M33D also neutralizes lipopolysaccharide and lipoteichoic acid, thus exerting anti-inflammatory activity by reducing expression of cytokines, enzymes and transcription factors (TNF-alpha, IL6, COX-2, KC, MIP-1, IP10, iNOS, NF-kB) involved in the onset and evolution of inflammatory processes [17].

By virtue of all these characteristics and its low toxicity [17], M33D and its analogue M33l/l are considered good candidates to fight endodontic and periodontal disease.

MATERIAL AND METHODS

Peptide synthesis

The peptides M33D (kkirvrlsa)4K2KβA-OH and M33i/l (kklrvrlsa)4K2KβA-OH were solid-phase synthesized by standard Fmoc chemistry using D-amino acids with a Syro multiple-peptide synthesizer (MultiSynTech,Witten, Germany). We used a TentaGel-PHB 4 branch βAla Wang-type resin (Rapp Polymere, Germany), which carries the branching core in L-form, Fmoc4-Lys2-Lys-β-Ala, as previously described [13]. Side-chain protecting groups were 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for R, t-butoxycarbonyl for K and t-butyl for S. The final product was cleaved from the solid support, deprotected by treatment with TFA containing triisopropylsilane and water (95/2.5/2.5) and precipitated with diethyl ether. Crude peptide was purified by reversed-phase chromatography on a Phenomenex Jupiter C18 column (300 Å, 10 mm, 250, 610 mm), using 0.1% TFA/water as eluent A and methanol as eluent B, in a linear gradient from 80% A to 20% A in 30 min. Final peptide purity and identity were confirmed by reversed-phase chromatography on a Phenomenex Jupiter C18 analytical

column (300 Å, 5 mm), M33D RT = 21 min, M33i/l RT = 22 min, and by mass spectrometry with a Bruker Daltonics ultraflex MALDI TOF/TOF), M33D M+(found) = 4682.36, M33i/l M+(found) = 4682.86.

Peptide structure prediction

De novo prediction of peptide tertiary structure was achieved with APPTEST [19] where a computational protocol combines the predictive power of neural networks with existing structural biology software programs XPLOR-NIH and CYANA. Neural networks were trained on experimentally obtained model structures from the PDB (protein data bank, https://www.rcsb.org/) to predict structural restraints, which are used in restrained molecular dynamics simulations to yield a final ensemble of structures.

Minimum inhibitory concentrations

The bacteria reported in Table 1 were used for conventional susceptibility testing experiments. M33D and M33i/l MICs were determined in triplicate on reference and clinical strains using a reference microdilution assay, performed according to the guidelines of the Clinical and Laboratory Standards Institute [20]. Briefly, strains were grown on Mueller-Hinton agar plates and a single colony for each strain was picked using a sterile cotton swab, streaked in sterile cation-supplemented Mueller-Hinton broth (Becton Dickinson, Franklin Lakes, NJ, USA) and measured with a densitometer (Densicheck, bioMèrieux, Marcy l'Etoile, France) up to 0.5 McFarland density. A total of 50 µL of each bacterial suspension was used to inoculate wells of a microtiter plate containing an equal volume of serial-doubling dilutions of the peptides, performed in the same suspension media used for bacterial inocula.

Assays were performed using a final bacterial inoculum of 5 x 10^4 CFU/well in a volume of 100 µL. MIC values were recorded after incubation of plates at 35°C for 18–20 h. Assays were performed in triplicate and the median MIC values were recorded.

Membrane interaction

Mid-log phase *E. faecalis* PE, resuspended at 1 x 10^8 CFU/mL in PBS-glc, was incubated in an orbital shaker at 37°C for 15 min. Then PI or SYTOx green dye was added to final concentrations of 5 µg/mL and 5µM, respectively. The suspension was mixed by short vortexing and 200 µL was added to the wells of a black 96-well plate (Optiplate). Samples were preincubated at 37°C with fluorescence measurements every minute for 5 min, or until readings stabilized. The plate was then ejected. Peptides (2x and 4xMIC) were added in duplicate wells. The plate was immediately returned to the reader to continue monitoring PI and SYTOx green (PI; λex = 535, λem = 617 nm; SYTOx green λex = 504, λem = 523 nm), every 1 min for 100–120 min.

Anti-biofilm activity

Biofilm inhibition and destruction activity were evaluated by two independent parameters: the biofilm prevention concentration (BPC) and the minimal biofilm inhibition concentration (MBIC) [21-22]. Briefly, for BPC, an overnight culture of *E. faecalis* PE, *E. faecalis* 51299 or *E. coli* TG1 bacteria was suspended to 0.8 McFarland standard in TSB (Tryptic Soy Broth) 0.25% glucose. This bacterial suspension (100 µL) was added to wells in triplicate in a 96-well U-bottom plate, then 100 µL M33D and M33i/l were added to the well with twofold serial dilution of each peptide.

Untreated bacteria were used as positive control. After 24 h of static incubation at 37°C, wells were washed thrice with PBS and fixed with PFA 4% in PBS (200 µL) for 30 minutes at room temperature. Wells were then washed 3 times and crystal violet (1% in water) was added and incubated for 30 minutes in the dark. Wells were again washed and the colour dissolved with ethanol:acetone 80:20. Colour was read at 595 nm. The BPC is the lowest concentration of an antibiotic that results in an OD595 nm difference of ≤10% in the mean of two control well readings.

For MBIC, the bacterial suspension (200 µL) of *E. faecalis* PE, *E. faecalis* 51299 or *E. coli* TG1, obtained as above, was added to wells in triplicate, in a 96-well U-bottom plate and incubated at 37°C for 24h. The supernatant was removed and 200 µL of M33D and M33i/l diluted in TSB were added to the wells in twofold serial dilutions of each peptide.

Untreated bacteria were used as positive control. After 14 h of static incubation at 37°C, wells were washed thrice with PBS and fixed with PFA 4% in PBS (200 µL) for 30 minutes at room temperature. Wells were then washed three times and crystal violet (1% in water) was added and incubated for 30 minutes in the dark. Wells were again washed and the colour dissolved with ethanol:acetone 80:20. Colour was read at 595 nm. The MBIC is the lowest concentration of an antibiotic that results in an OD595 nm difference of ≤10% of the mean of two control well readings.

Dentin slice model

Extracted human molars and premolars were obtained from patients according to a protocol approved by the Ethics Committee of the Osaka University Graduate School of Dentistry (no. H25-E23).

Teeth were sliced with a diamond saw at low speed. Slices were then treated with 40% phosphoric acid for 1 min, followed by 5.25% sodium hypochlorite for 10 min in an ultrasound bath.

The prepared dentin slices were placed in a 24-well plate and challenged with a suspension of *E. faecalis PE* bacteria (OD=0.8), incubated at 37°C for 1 h at 110 rpm. Supernatant was removed and the slices were washed three times with PBS or a solution of the peptides in PBS at a concentration of 10 uM. Dentin slices were then incubated at 37°C for 1h in TSB. The supernatant suspension was serially diluted and plated in TSB-agar. CFU were counted after incubation overnight at 35°C:

LPS-peptide binding assay

M33D and M33Di/l were diluted to working concentrations (10 µg/ml; 5 µg/ml; 1µg/ml) in carbonate buffer (pH 9) and a 96-well ELISA strip plate

was coated with 100 µl per well. The negative controls were uncoated wells. The plate was sealed and incubated overnight at 4°C. Each well was aspirated and washed three times with PBS + 0.05% Tween 20 and with PBS. The plate was blocked by adding 400 µl/well PBS and milk 3% and incubating for 2h at 37°C. LPS-Bio (Aurogene srl) was diluted in PBS and BSA 0.3% to a working concentration of 5 µg/ml. The negative control contained only PBS and BSA 0.3%. The plate was incubated in the dark at 30°C for 30 minutes. After washing, 100 µl/well Streptavidin-POD (Sigma Aldrich) diluted 1:500 in PBS and milk 0.3% was added and incubated in the dark for 30 minutes at 30°C. After washing, 150 µl/well of substrate solution was added, the reaction was stopped with 50 µl/well HCl 1M and the plate was read at 450 nm and 650 nm using a microplate spectrophotometer (Multiskan, Thermo Scientific).

Human cardiac fibroblast modulation

Human cardiac fibroblasts (HCF) (Innoprot P104452) were plated in a standard 96-well plate at 500,000 cells/ml. After 24-hour incubation at 37°C, HCF were treated with LPS 2 µg/ml and the peptides at 16 µg/ml and left overnight at 37°C. At the same time a DuoSet ELISA plate (R&D Systems Inc.) was prepared according to the manufacturer's instructions, adding 100 µl/well of the capture antibody. After 24h, the DuoSet plate was washed and blocked with 1% BSA in PBS. The HCF supernatant was collected and centrifuged for 5 minutes at 1200 rpm and added to the DuoSet plate at 100 µl/well and incubated for 2h at RT. After washing, the detection antibody was added and left for 2h at RT. Finally, streptavidin-peroxidase was used for detection and the plates were read at 450 nm using a microplate spectrophotometer (Multiskan, Thermo Scientific). The IL6 concentration was calculated from a standard curve that was linearized by plotting the log of the six concentrations versus the log of the antibody.

Eukaryotic cell viability assay

RAW 264.7 cells were seeded in 96-well plates (5 x 10^3 per well) and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. They were then treated with 100 µL fresh medium containing the peptides at different concentrations for 24 h. Then 20 µl MTT (5 mg/mL) was added to each well and the plate was incubated at 37°C for 3 h. Finally, 120 µL HCl 4 mM in isopropanol was added to each well to dissolve the formazan crystals. Optical density was measured with a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Cell viability was calculated by comparing the values of treated with those of untreated cells. EC50 was calculated using GraphPad Prism 5.03 software.

Haemolytic activity

Whole human blood in EDTA was centrifuged (x1100 g) for 10 min. Red blood cells diluted 1:100 in physiological solution (0.9% NaCl) were incubated for 24 h at 37° C with serial dilutions of the peptides from 1.25 to 340 uM. The absorbance of the supernatants was determined in a 96-well plate at 490 nm with a microplate reader. Data for 100% haemolysis was obtained by adding 0.1% TritonX-100 in water to the cells. The negative control was with physiological solution. The haemolysis rates of the peptides were calculated with the following equation: (%) = (A peptide—A physiological solution)/(A triton—A physiological solution) 100%; where A = absorbance.

Statistical analysis

The data was plotted and analysed using Excel (Microsoft, Redmond,WA, USA) and GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). It is reported as means ± SD, where relevant. Experiments were repeated at least twice.

RESULTS

M33D and M33i/l

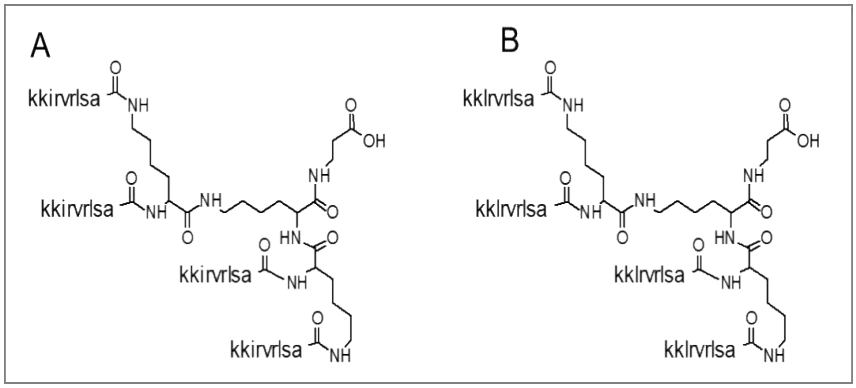

Figure 1. Structure of the branched antimicrobial peptides, M33D (A) and M33i/l (B).

M33D and M33i/l are synthetic peptides built on a three-lysine core that allows four sequences on the same scaffold. The amino acids used for the sequences are all of the D-series, except the three lysines of the core. M33i/l carries a leucine in the place of the isoleucine of its parent compound M33D. Leucine, having one less stereocenter than isoleucine, gives fewer stereochemistry by-products when transformed into a Fmoc-derivative and included in the amino acid sequence. It is therefore more affordable industrially for future development.

Minimal inhibitory concentration (MIC)

MICs of M33D and M33i/l were determined against strains of different Gram-negative and Gram-positive bacterial species (Table 1). M33D was also tested against a *Corynebacterium,* two *Streptococci* and three *Candida* strains.

TABLE 1 MIC of M33d and M33i/l against bacterial strains representative of various pathogenic species, including MDR strains of clinical origin.

The strains tested included reference strains (indicated) and clinical isolates (mostly with a MDR phenotype). Relevant resistance traits and resistance mechanisms are indicated. FQ^r resistant to fluoroquinolones; AG^r resistant to amino-glycosides (gentamycin, amikacin and/or tobramycin); ESC^r resistant to expanded-spectrum cephalosporins; NEM^r resistant to carbapenems (imipenem and/or meropenem); ESBL extended spectrum-β-lactamase; MBL metallo-β-lactamase; MR methicillin-resistant; VAN ^{i} vancomycin-intermediate. [a]Post endodontic treatment isolate. [b]Determined with standard antiobiogram. *values from [16]. Both M33D and M33i/l showed MIC against Gram-negative species with a range of activity between 8 and 32 µg/ml for M33D and between 4 and 32 µg/ml for M33i/l. Regarding Gram-positive bacteria, M33D showed good activity against *Staphylococcus aureus* and *epidermidis*, ranging from 4 to 8 µg/ml, including methicillin (*S. aureus* USA300, *S. aureus* 3851) and vancomycin (*S. aureus* 3851) resistant strains. *Enterococcus faecalis* and *faecium* were susceptible to M33D in the 4-16 µg/ml range. *Streptococcus* species *pneumoniae* and *pyogenes* were susceptible to M33D in the 4-16 ug/ml range and *Candida* species in the 24-64 µg/ml range. The antimicrobial activity of M33i/l showed improved activity against the *Pseudomonas aureoginosa* strains tested (Table 1). *E. coli, K. pneumonia, Enterococcus and Staphylococcus* species tested can be considered equally susceptible to M33i/l and M33D. The MICs are in line with previously reported well-known antimicrobial peptides [23].

Mechanism of action of M33-D and M33i/l against *Enterococcus faecalis*

Most natural antibacterial peptides impair membrane function, seriously challenging the bacteria cell. Membrane-targeting peptides can increase permeability to small ions or larger molecules and cause extensive membrane damage [24].We visualized the kinetics of pore formation using membrane-impermeable fluorescent dyes such as propidium iodide (PI) and SYTOX green [25-26] in a *E. faecalis* post-endodontic treatment isolate (PE). Their fluorescence increases on binding to nucleic acids, which only happens when the cytoplasmic membrane is critically damaged.

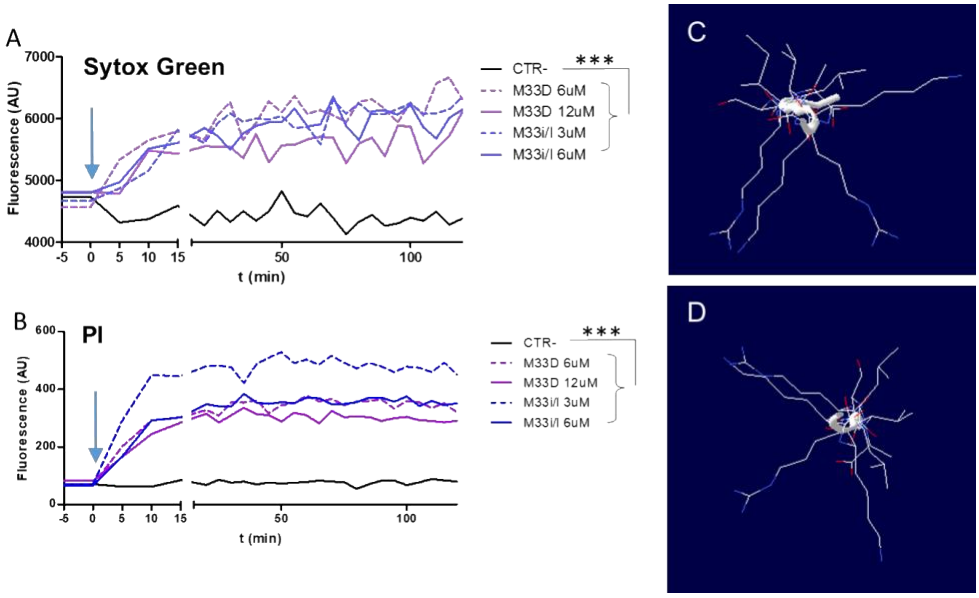

Figure 2. Observations on peptide mechanism of action. Membrane permeabilization (A) Sytox Green and (B) PI. Experiments were performed with 10^8 CFU/mL *E. faecalis* PE in PBS-glucose. The arrow indicates the time when the peptides were added. The peptides were used at 2- and 4-fold their MIC. *** $p<0.0001$ for both peptides and both concentrations. De novo prediction of peptide tertiary structure by APPTEST for the linear analogues of M33D (C) and M33i/l (D).

Figure 2 A and B shows that peptides M33D and M33i/l induce rapid permeabilization of the membrane within 15 minutes of their addition at 2- and 4-fold the MIC. The results indicate that the two peptides can induce pore formation in the *E. faecalis* PE membrane within 10-15 minutes.

A prediction of peptide tertiary structure was obtained with the computational protocol of APPTEST [16] using the linear analogues of M33D and M33i/l (Figure 2 C and D). In both peptides, the sequence of amino acids takes a random configuration, i.e. linear instead of helical; the top view of their structure shows overall amphipathic structure, where the aliphatic non-polar side chains of leucine, isoleucine, valine and alanine arrange on opposite sides with respect to polar cationic lysines and arginines. The amphipathic characteristic underlies the embedding property of the peptide in the cell

membrane, the first step in impairing membrane function that kills the bacteria.

Inhibition of biofilm formation

In biofilms, bacteria produce proteins and polysaccharides and grow as multicellular aggregates in an extracellular matrix that shelters their cells from environmental insults and host defences. Biofilms are also more resistant to antimicrobial agents due to the physical barrier generated by the matrix and the state of dormancy of the bacteria [27-29]. Microbial biofilms are regarded as a primary cause of periodontitis in teeth with infected root canals [30]. *E. faecalis* is the most prevalent species [31] in persistent intra-radicular infections, facilitated by its facultative anaerobe property. *E. faecalis* biofilm is particularly resistant and poses a major obstacle to endodontic disinfection of root canals.

M33D and M33i/l were tested for their anti-biofilm activity against *E. faecalis* PE clinical isolate and reference strain and also against *E. coli* TG1 reference strain. Biofilm inhibition activity was evaluated on the basis of two parameters: the biofilm prevention concentration (BPC) and the minimal biofilm inhibition concentration (MBIC) [21]. The BPC is lowest concentration of peptide that results in 10% lower biofilm formation compared to untreated controls. The biofilm was measured after removing planktonic cells by washing the wells, previously incubated for 24h with the bacteria and the peptide. MBIC is lowest concentration of peptide that results in a 10% reduction in preformed biofilm compared to untreated controls. A 24h biofilm was challenged with serial dilutions of the peptides for 14h. Then after removal of planktonic cells, the residual biofilm was measured. Crystal violet absorbance at 585 nm was used to measure biofilm in the case of both parameters. The BPC of M33D was twice the MIC against both strains of *E. faecalis*. The BPC of M33i/l was equal to the MIC of the post-endodontic isolate and eight times the MIC of the reference strain. The BPC of both peptides against a reference strain of *E. coli* was equal to the MIC, i.e. 1.5

uM. The MBIC of *E. faecalis PE* was 1.5 μM, equal to the MIC of both peptides and in the 1.5-6 μM range for *E. faecalis* reference strain. The MBIC of *E. coli* was below 15 μM, the minimum concentration tested.

The results are particularly promising when compared to the MBICs of ampicillin and linezolid against *E. faecalis* which are 2048 and 1024 times the MIC, respectively [32].

Inhibition of bacterial regrowth on dentin slices by washing with the peptides

The gold standard for the elimination of intra-canal bacteria is irrigation of the root canal with antiseptic solutions [30]. The most common is sodium hypochlorite, though it often does not completely eradicate bacteria and may therefore be followed by relapse of infection. Dentin slices were used as a model of dental preparation before restoration to reduce bacterial bioburden and improve the outcome of bonded restorations [33]. Teeth slices were obtained from extracted human molars and premolars using a diamond saw. The dentin discs were sterilized, then infected and finally washed three times with a solution of the antimicrobial peptides at a concentration of 10 μM.

Washing with the two peptides reduced the bacterial burden to nearly one twentieth with respect to control and to a lesser extent with respect to PBS wash, but still statistically significant (Figure 3). Irrigation, being a local treatment, allows use of a much higher concentration of M33D and M33i/l and also to alternate washes with antimicrobial peptides and hypochlorite solution, as would be feasible and realistic in a typical root canal treatment.

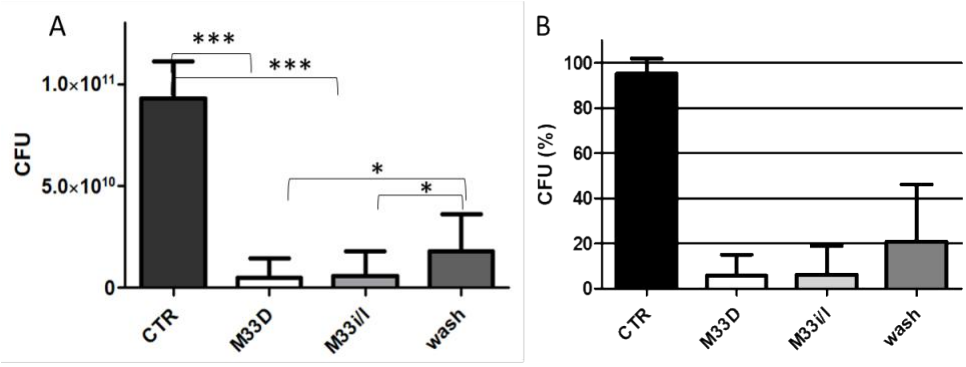

Figure 3. Inhibition of bacterial regrowth expressed in CFU (A) and as percentage (B) after washing dentin slices with peptides; ***p<0.0001; *p<0.0188 M33D; * p=0.0423 M33i/l, CTR n=5, M33D and M33i/l n=20, washes n=10.

Modulation of human cardiac fibroblast activity

M33D was previously shown to neutralize lipopolysaccharide (LPS) and lipoteichoic acid (LTA) and also to consistently reduce expression of mediators of inflammation such as TNF-alpha, IL6, COX-2, KC, MIP-1, IP10, iNOS and NF-kB [17]. This immunomodulatory activity is initiated by the peptides capturing LPS. As shown in Figure 4A and B, M33D and M33i/l could bind LPS-biotin with dose-dependent linearity (Figure 4 A and B). This ability of the peptides to capture LPS also proved to underlie human cardiac fibroblast inhibition of IL6 release triggered by LPS.

Cardiac fibroblasts contribute to cardiac physiology with many functions, such as insulation of the conduction system and vascular maintenance [34]. They promote a response to insult in association with the immune system. Bacteria can trigger fibroblasts to produce inflammatory mediators (cytokines, chemokines and growth factors) that recruit inflammatory cells from the circulation, amplifying the inflammatory process and inducing heart dysfunction [35-38].

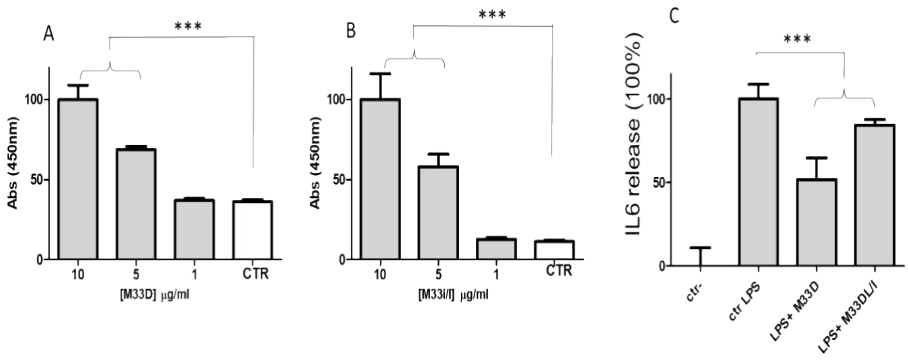

Figure 4. A) M33D and B) M33i/l binding to LPS (n=6, for all groups, ***p<0.0001). C) HCF release of IL6 in the presence of M33D and M33i/l (n=3, ***p<0.0001). Maximum IL6 release was 2.29 µM calculated from a linearized standard curve.

Human cardiac fibroblasts were stimulated for 24h with LPS with or without M33D and M33Di/l peptides. The release of IL6 was reduced by both peptides: by 50% in the case of M33D and 35% in the case of M33Di/l.

Cytotoxicity of M33D and M33i/l in eukaryotic cells

Cytotoxicity to eukaryotic cells was tested in RAW264.7 murine macrophages with 24-h incubation at 37°C. M33D and M33i/l showed EC50s of 3.0 x 10^{-5} M and 1.8 x 10^{-5} M, respectively, both one order of magnitude higher than the average MIC in the different species.

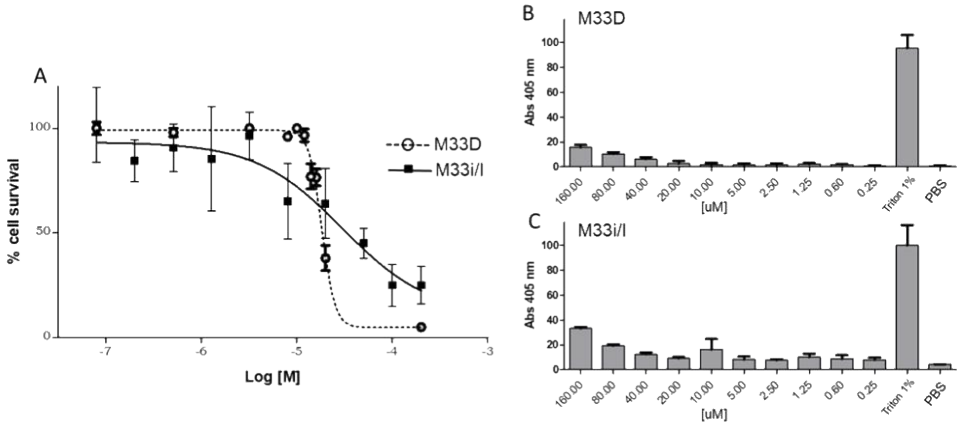

Figure 5. Cytotoxic and haemolytic effect of M33D and M33i/l. A) RAW 264.7 were incubated with increasing concentrations of peptides for 24 hours at 37°C. Cell viability is reported as a percentage of the untreated cells (n=3). B) and C) Haemolytic activity of M33D and M33i/l is reported as a percentage ± SD of 100% obtained with triton 1% in PBS after incubation for 1h at 37°C [29]. M33D and M33i/l did not show relevant haemolytic activity when incubated with red blood cells for 1 h at 37°C and compared to triton at 1% in PBS.

Conclusions

Bacteria of the human oral microbiota can occasionally turn into pathogens. Among these, *Enterococci* are major players, contributing to severe acute and chronic infections, both of which are atherogenic and lead to cardiovascular disease. M33D and M33i/l proved to be active against a number of different

Gram-positive and Gram-negative bacteria, in planktonic form and also in biofilm, including *E. faecalis* isolated during endodontic treatments. Importantly, M33D and M33i/l show immunomodulatory activity, crucial for the healing of oral diseases, and especially for avoiding onset of atherosclerotic cardiovascular disease. The use of antimicrobial peptides to irrigate tooth cavities and in medications is a new field of development that promises tools to fight dental infections and prevent their severe consequences, while at the same time protecting standard antibiotics against new outbreaks of antimicrobial resistance.

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6

6. Cumulative effect of perceived stress and sleep quality on periodontitis severity. Results from a University-based cross-sectional study.

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1 Introduction

Stress and lack of adequate rest are among the major issues of the modern lifestyle [1]. As for the former, stress responses can be assessed through a variety of tools, evaluating either subjective, objective or physiological parameters [1]. In particular, perceived stress refers to a condition that is perceived as threatening or uncontrollable by the subject [2]. High levels of stress affected around 20% of Americans in 2020 [3]. Indeed, stress levels are strongly affected by educational, cultural and socio-demographic characteristics [4]. Stress affects health through a network of biological mechanisms and behavioral changes; individuals may respond to stress by adopting unhealthy behaviors, such as exercising less frequently, comfort eating, drinking or smoking excessively [4].

Another important aspect of the modern lifestyle is the lack of adequate sleep. Epidemiological evidence in the American population demonstrated that around 20% of individuals reported suffering from chronic sleep disorders [5].

Poor sleep quality was demonstrated to lead to increased levels of inflammatory markers and to contribute to the occurrence and progression of several inflammatory diseases [6].

High levels of perceived stress and poor sleep quality are recognized as modifiable risk indicators for periodontitis and several other non-communicable diseases (NCDs) [7–9], together with other aspects of the patient's lifestyle (*e.g.* smoking, unhealthy diet, lack of physical exercise etc.) [10–12]. Given the huge impact of the modifiable risk factors on the occurrence and progression of several NCDs, a lot of research focused on the implementation of a multifactorial approach targeted at improving various aspects of the patient's lifestyle, including stress and poor sleep quality [13,14].

While some evidence is present on the independent impact of stress and sleep quality on periodontal health [15,16], no data is available regarding the cumulative effect of high perceived stress and poor sleep quality on the occurrence, severity and rate of periodontitis progression. The hypothesis leading up to the study was that the detrimental effects of both high stress and poor sleep quality may accumulate and thus contribute to a worse periodontitis phenotype.

Therefore, the aim of the present study was to evaluate the cumulative effect of perceived stress and sleep quality on the biometric and inflammatory periodontal parameters of a University-based cohort of individuals.

2 Materials and methods

2.1 *Study design*

The present study is reported according to the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) guidelines for cross-sectional studies [17]. The research protocol was approved by the local ethics committee (protocol number: 18993/2021) and was registered on Clinicaltrials.gov (NCT04771949).

2.2 *Setting and participants*

All consecutive patients attending the Dentistry Department at the University Hospital of Siena were screened between January 2021 and August 2021; the inclusion criteria were:

- age between 18 and 70 years old;
- ability and willingness to give informed consent.

The exclusion criteria were:

-pregnancy or lactation;

-periodontal therapy performed in the last 12 months;

-administration of antibiotics within the last 6 months;

-inability to communicate effectively in Italian.

Individuals were included in the study after they read and signed the written informed consent, in accordance with the Declaration of Helsinki.

2.3 *Variables*

2.3.1 *Socio-demographic characteristics*

Socio-demographic characteristics, including patients age, gender, smoking and oral hygiene habits, occupation and education level, were registered.

Moreover, data regarding the presence of familiarity as well as any comorbidity affecting susceptibility to periodontitis were recorded. The Body Mass Index (BMI) was computed as weight (kilograms)/height (meters^2). The assessment methods of socio-demographic characteristics are reported in the Supplementary Appendix.

2.3.2 *Perceived stress assessment*

The Italian version of a validated 10-item questionnaire to measure patients' level of perceived stress (IPSS-10) was administered by two examiners, following the structured questions and explanations provided by the questionnaire [18]. The instrument had 10 questions with response options ranging between 0 (never) and 4 (very often). All questions were negatively stated, except for 4 questions that were positively stated (items 4, 5, 7 and 8). The sum scores were calculated after reversing the positive items' scores and then summing up all scores. Total scores ranged between 0 and 40; the higher the score, the higher the level of perceived stress. Finally, the median IPSS-10 score was considered in order to stratify participants into two subgroups: high (IPSS-10≥13) and low perceived stress (IPSS-10<13).

2.3.3 *Sleep quality assessment*

Sleep quality was assessed using the validated Pittsburgh Sleep Quality Index (PSQI) questionnaire [2]. The Italian version of the PSQI was administered by two examiners, who asked structured questions and gave the explanations provided by the questionnaire. The questionnaire included 7 domains, each one assessed with a 0 to 3 scoring system; the final scores ranged between 0

and 21, with higher values indicating worse sleep quality. Participants with a total score of 5 or higher were classified as having "poor sleep quality", vice versa for those with lower scores (classified as having "good sleep quality") [19].

2.3.4 *Periodontal examination*

All participants received a full periodontal chart by two trained and calibrated examiners (unweighted *kappa* score of 0.98). Examiners were calibrated by performing a full periodontal chart on two non-study subjects affected by periodontitis; the examiner was considered reproducible if an agreement of at least 95% of Clinical Attachment Level (CAL) (with maximum a 2 mm difference) between two repeated measurements was recorded. Periodontal Probing Depth (PPD), gingival Recession (REC), plaque [20] and Bleeding on Probing (BoP) [21]were recorded with a standardized periodontal probe* six sites per tooth, third molars excluded. Whenever the Cementum Enamel Junction (CEJ) was subgingival, CAL was measured as the difference between PPD and the distance between the free gingival margin and the CEJ. The presence of furcation involvement was recorded according to the classification of Hamp [22]; the classification of Miller [23] was used to record tooth mobility.

* UNC 15 probe, HuFriedy Group, Chicago, Illinois, United States

A periodontitis case was defined whenever interdental CAL was detectable at ≥2 non-adjacent teeth, or whenever buccal or oral CAL≥3mm with pocketing (PPD>3mm) was detectable at ≥2 teeth [24]. Periodontitis severity, complexity of management and extent of distribution were assessed with the Staging [25];

the Grading was used in order to assess the rate of disease progression [25]. Whenever possible, the Grade was assigned with direct evidence of disease progression (*i.e.* longitudinal data of radiographic bone loss or CAL over 5 years); whenever these data were not available, then indirect evidence was used (case presentation or % bone loss/age). As for the Grade modifiers, smoking status was self-reported, while the diagnosis of diabetes was ascertained by checking the patient's medical report; glycohemoglobin levels were taken into account only when available [25].

2.4 *Statistical analysis*

2.4.1 *Sample size calculation*

Sample size was calculated considering the prevalence of periodontitis in the reference cohort at 37.3% [26] and its value in the study cohort as 10% higher. Considering α=0.05 and β=0.80, the computed sample size was of 185 subjects. Given a non-response rate of 20%, the inclusion of 235 participants was planned.

2.4.2 *Descriptive and inferential statistics*

Statistical analysis was performed through an *ad hoc* software† setting the level of significance at 5%. Continuous variables were reported as Mean with 95% Confidence Interval; categorical data were expressed as number of observations (proportion). After verification of data distribution, Kruskal-Wallis and Fisher's exact tests were used to compare patients' characteristics according to perceived stress, sleep quality and their possible combination

(low stress and good sleep quality, low stress and poor sleep quality, high stress and good sleep quality, and high stress and poor sleep quality).

† STATA BE, version 17, StataCorp LP, Texas, United States

2.4.3 *Logistic regression models*

Univariate and multivariate logistic regression analyses were performed to compute the association between periodontitis, periodontitis stage III/IV, and periodontitis Grade C according to perceived stress, sleep quality and each domain of the PSQI; it was expressed as crude and adjusted odds ratios (ORs). ORs were adjusted for parameters that could affect periodontitis phenotype (*i.e.* age, gender, smoking, and brushing frequency); these parameters were selected according to external knowledge.

3 Results

3.1 *Participant characteristics*

A total of 235 participants were included in the present study. All individuals examined for eligibility accepted to participate, were enrolled in the study and then included in the analysis. Subjects' characteristics are reported in Table 1; details of the study population according to oral health status are reported in a previous publication [11]. The mean age was 53.9[52.1,55.8] years and the mean BMI was 25.5[24.9,26.1]. The majority of subjects were females (57.9%) and the proportion of smokers was 25.9%. Around 20% of subjects were affected by at least one comorbidity. Moreover, 40% of participants

were affected by Stage 3 periodontitis, while 47.2% and 29.4% were diagnosed with Grade B and C periodontitis, respectively.

3.2 *Outcome data*

3.2.1 *Perceived stress and periodontitis*

High perceived stress was significantly associated with a higher severity (staging) and progression (grading) of periodontitis (Table 1). Other periodontal variables (*i.e.* mean CAL, mean PPD, %PPD>4mm, %PPD 5-6mm, %PPD >6mm, tooth mobility, number of bleeding pockets, FMBS, teeth lost for periodontal causes) resulted to be significantly worse in subjects with high compared to low perceived stress (Table 1). FMPS as well as domiciliary plaque control habits were comparable across subgroups of perceived stress (p=0.56) (Table 1). Moreover, high perceived stress was significantly associated with higher odds of both periodontitis (OR=4.8, 95% CI 1.8-12.9, p=0.00) (Table 3) and stage III/IV periodontitis (OR=2.5, 95% CI 1.3-4.6, p=0.00) (Table 4), but not with Grade C (OR=0.9, 95% CI 0.5-1.8, p=0.79), after adjusting for age, BMI, gender, smoking and brushing frequency (Table 5).

3.2.2 *Sleep quality and periodontitis*

Subjects with poor and good sleep quality were almost equally distributed among the population (48.1% and 51.9%, respectively), but the former category presented a significantly higher age compared to the latter (p=0.00) (Table 1). Moreover, poor sleep quality was significantly associated with a

higher severity (staging) and progression (grading) of periodontitis (Table 1). Other periodontal variables (*i.e.* mean PPD, %PPD>4mm, %PPD 5-6mm, %PPD >6mm, furcation involvement, tooth mobility, number of bleeding pockets, teeth lost for periodontal causes) were reported to be significantly worse in subjects with poor compared to good sleep quality (Table 1). Domiciliary plaque control habits, namely frequency of toothbrushing and interdental cleaning, and toothbrush type, were comparable across subgroups of sleep quality (p=0.99). Moreover, poor sleep quality was associated with higher odds of periodontitis, but not after adjustments (OR=2.4, 95% CI 0.9-6.1, p=0.07) (Table 3). Conversely, adjusted ORs revealed a statistically significant association between poor sleep quality and stage III/IV periodontitis (OR=2.2, 95% CI 1.2-4.1, p=0.01) (Table 4), and Grade C (OR=1.4, 95% CI 1.0-2.8, p=0.04) (Table 5). None of the sleep components were significantly associated with periodontitis, while a high sleep latency and a sleep duration less than 8 hours resulted in significantly positive adjusted ORs for both stage III/IV periodontitis (OR=3.1, 95% CI 1.1-8.7, p=0.04 and OR=2.4, 95% CI 1.1-3.6, p=0.02, respectively) and Grade C (OR=2.9, 95% CI 1.1-7.8, p=0.03 and OR=1.3, 95% CI 1.0-3.0, p=0.04, respectively) (Tables 3-5).

3.2.3 *Combining perceived stress and sleep quality*

No significant differences in socio-demographic characteristics were found across the 4 subgroups (Table 2). Around 30% of participants belonged to the "high stress, poor sleep quality" subgroup and the "low stress, good sleep quality" subgroup, respectively; fewer subjects belonged to the other three

categories. Cases of periodontitis stages III and IV significantly increased from the "low stress, good sleep quality" (23.9% and 5.6%, respectively) to the "high stress, poor sleep quality" (52.1% and 16.9%, respectively) subgroup. The proportion of subjects who reported having lost teeth for periodontitis almost tripled when shifting from the "low stress, good sleep quality" (14.5%) to the "high stress, poor sleep quality" (46.1%) subgroup (p=0.00) (Table 2). Subgroups "high stress, poor sleep quality" and "high stress, good sleep quality" resulted in significantly positive adjusted ORs for periodontitis (OR=9.2, 95% CI 1.8-45.1, p=0.00, and OR=4.6, 95% CI 1.4-15.5, p=0.01, respectively) (Table 3); significantly higher adjusted odds for Stage III/IV periodontitis were demonstrated for all subgroups (p<0.05) (Table 4). Conversely, no significant association was detected between all combinations of stress and sleep quality, and Grade C periodontitis (p>0.05) (Table 5).

4 Discussion

4.1 *Summary of findings*

In the current study, subjects with high perceived stress as well as those with poor sleep quality were found to have significantly worse periodontal parameters compared to those with low perceived stress and high sleep quality, respectively. Nonetheless, plaque control was comparable across subgroups of perceived stress and sleep quality. After adjusting for confounders, the odds of stage III/IV periodontitis were more than two times higher in subjects with high *versus* low perceived stress, and in those with

poor *versus* good sleep quality. Poor sleep quality, but not high perceived stress, was associated with significantly higher odds of having rapidly progressing periodontitis (Grade C). Moreover, a cumulative effect between high perceived stress and poor sleep quality was demonstrated, leading to 9-time increased odds of having periodontitis compared to the combination between low perceived stress and good sleep quality.

4.2 *Perceived stress and periodontitis*

The present study highlighted a significant association between perceived stress and periodontitis. Subjects with high perceived stress, defined as having a IPSS-10 score of 13 or higher, were 90% more likely to have periodontitis, and 80% more likely to have severe forms of periodontitis. While perceived stress was associated with disease occurrence and severity, it was not associated with the rate of disease progression (Grading of periodontitis). There are many studies investigating the relationship between stress and periodontitis but, since stress responses can be assessed in various ways, only some of them investigated perceived stress [15,27,28]. Results of the current investigation are consistent with those obtained by a previous report conducted on 621 Brazilian individuals [15]. A significant association between perceived stress and oral health status was found with the two different case definitions employed for periodontitis, *i.e.* presence of at least one site with PD≥4mm or with CAL≥5mm, with the presence of BoP [15]. Nonetheless, the magnitude of association was weaker than what was found in the present study maybe due to the different definition used to identify periodontitis cases [25]. The association between stress and periodontitis may be boiled down to

mainly two pathways: direct biological impact on the immune and endocrine system, and behavioral changes [1]. As for the direct biological impact, stress triggers an increase in neuroendocrine hormones, such as glucocorticoids and catecholamines which, in turn, exert suppressive effects on the immune system, by reducing lymphocyte proliferation, antibody production and natural killer cell activity [29]. Consequently, subjects with chronic stress tend to be more prone to infections. Moreover, stress can affect oral health also by inducing behavioral changes, such as smoking, excessive alcohol consumption and comfort eating, which are also influenced by socioeconomic factors [4]. In the current study, subjects with high and low perceived stress had no significant differences as for smoking status and all socioeconomic factors, except for the educational level. Given that our estimates for the association between stress and periodontitis were adjusted also for the educational level, a direct biological impact of stress on the periodontium can be speculated.

4.3 *Sleep quality and periodontitis*

Poor sleep quality was found to be associated with higher severity and rate of disease progression of periodontitis. Results from the current study are consistent with those from a previous study investigating the relationship between sleep quality, as assessed with the PSQI, and periodontitis [16]. Indeed, they demonstrated a significant association between staging and grading of periodontitis with PSQI, and with each of its components. A comparison of the magnitude of association between the previous and the current study could not be performed since the outcome measures as well as the thresholds used for variables categorization differ. Substantial evidence supports the

biological plausibility of the association between poor sleep quality and periodontitis; this relationship seems to be bidirectional [30–33]. Indeed, poor sleep quality was found to be associated with higher markers of systemic inflammation which may have an effect on the development of periodontitis [34,35]; in turn, the presence of a state of systemic inflammation, which can be triggered by periodontitis [36], may negatively impact sleep quality. Furthermore, among the components of sleep quality, a high sleep latency (*i.e.* >30 minutes), as well as a short sleep duration (*i.e.* less than 8 hours/night) led to significantly higher odds of having stage III/IV and Grade C periodontitis. Both sleep latency and sleep duration were previously associated with higher levels of systemic inflammatory markers [35,37]; nonetheless, few data is available regarding their relationship with periodontitis. As for the former, Karaaslan et al. (2019) [16] demonstrated a significantly positive association between higher periodontitis stage and grade, and a higher sleep latency, consistently with the current study. As opposed to the present findings, evidence from a nationally representative study observed an inverse relationship between the number of hours slept and periodontitis; the longer the sleep duration, the higher the odds of periodontitis [32]. Nonetheless, the current study failed to investigate the effects of long sleep duration on periodontal health due to the categorization used in the questionnaire.

4.4 *Cumulative effect of sleep quality and perceived stress*

The combination of high perceived stress and poor sleep quality was associated with worse periodontal parameters, irrespective of socio-

demographic characteristics and plaque control. Therefore, results from the current study suggest the presence of a cumulative effect of sleep quality and perceived stress on periodontitis occurrence and severity. In fact, a lifestyle characterized by high perceived stress and poor sleep quality led to 9-time increased odds of periodontitis, and more than 4-time increased odds of having severe forms of periodontitis (stage III/IV). To date, no previous study evaluated the combined effect of perceived stress and sleep quality on periodontal health. On the grounds of the above mentioned considerations, it can be hypothesized that the suppressive action on the immune functions of high perceived stress and the pro-inflammatory systemic action of poor sleep quality exert a cumulative effect on the periodontium, which is conducive to an increased susceptibility to periodontitis as well as disease severity.

4.5 *Strengths and limitations*

This is the first study investigating the cumulative effect of perceived stress and sleep quality on periodontal health. Assessments of perceived stress and sleep quality were carried out using reliable and validated tools for the selected sample; indeed, the validity and reproducibility of each questionnaire for the Italian population was previously demonstrated [2,38]. Altogether, these factors significantly contribute to the internal validity of the study. Nonetheless, some limitations should be taken into account. First of all, the cross-sectional design does not allow for an evaluation of the temporality between the cause and the effect and, as such, reverse causality could not be properly investigated. Moreover, no molecular parameters, *e.g.* inflammatory markers or stress hormones, were collected in order to support the biological

plausibility of such association. Secondly, given the lack of studies regarding the combined effect of stress and sleep quality on periodontal health, the sample size calculation was instead performed based on the prevalence of periodontitis; therefore, the potential lack of power could not be ruled out. Furthermore, all participants lived in the urban or suburban areas nearby Siena (Italy), where the vast majority of adults is Caucasian; hence, any variability in the outcome related to ethnicity may not have been detected. Moreover, given that the study population was selected among patients coming to a public University Hospital, the risk of selection bias could not be excluded. Overall, these factors may reduce the generalizability of the study.

5 Conclusion

The present study demonstrated that individuals conducing a lifestyle characterized by high perceived stress and poor sleep quality have 9-time increased odds of having periodontitis and are 80% more likely to have severe forms of periodontitis (stage III/IV). A high sleep latency and a short sleep duration (less than 8 hours/night) are associated with an increased severity and rate of periodontitis progression.

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Variable	Overall	Perceived Stress			Sleep Quality		
		High	Low	*p*-value*	Poor	Good	*p*-value*
	n=235	n=122	n=113		n=113	n= 122	
Age	53.9 [52.1, 55.8]	55.8±12.9	51.8±16.2	0.07	57.5±12.6	50.5±15.7	***0.00***
BMI	25.5 [24.9, 26.1]	25.3±4.2	25.7±5.4	0.93	25.9±5.1	25.1±4.5	0.22
Gender, females	136 (57.9%)	73 (59.8%)	63 (55.8%)	0.31	72 (63.7%)	64 (52.5%)	0.08
Occupation							
Unemployed	42 (17.9%)	25 (20.7%)	17 (15.1%)		18 (15.9%)	24 (19.8%)	
Employed	133 (56.8%)	64 (52.9%)	69 (61.1%)	0.41	61 (53.9%)	72 (59.5%)	0.24
Retired	59 (25.2%)	32 (26.5%)	27 (23.9%)		34 (30.1%)	25 (20.7%)	
Education							
Elementary/middle school	69 (29.5%)	44 (36.1%)	25 (22.3%)		36 (31.9%)	33 (27.3%)	
High school	107 (45.7%)	55 (45.1%)	52 (46.4%)	***0.02***	53 (46.9%)	54 (44.6%)	0.45
College or more	58 (24.8%)	23 (18.8%)	35 (31.3%)		24 (21.3%)	34 (28.1%)	
Smoking							
Never	107 (45.5%)	53 (43.4%)	54 (47.8%)		51 (45.1%)	56 (45.9%)	
Former	67 (28.5%)	37 (30.3%)	30 (26.5%)	0.77	32 (28.3%)	35 (28.7%)	0.99
Smoker	61 (25.9%)	32 (26.2%)	29 (25.7%)		30 (26.6%)	31 (25.4%)	
Familiarity for periodontitis, yes	87 (37.1%)	53 (43.4%)	34 (30.1%)	***0.02***	43 (38.1%)	44 (36.1%)	0.79
Diabetes							
No	180 (76.6%)	92 (75.4%)	87 (76.9%)		80 (70.8%)	99 (81.2%)	
Familiarity	44 (18.7%)	25 (20.5%)	19 (16.8%)	0.67	24 (21.2%)	20 (16.4%)	0.09
Yes	11 (4.7%)	5 (4.1%)	7 (6.2%)		9 (7.9%)	3 (2.5%)	
Rheumatoid arthritis							
No	198 (84.3%)	103 (84.4%)	95 (84.1%)		93 (82.3%)	105 (86.1%)	
Familiarity	18 (7.7%)	10 (8.2%)	8 (7.1%)	0.89	9 (7.9%)	9 (7.4%)	0.64
Yes	19 (8.1%)	9 (7.4%)	10 (8.9%)		11 (9.7%)	8 (6.6%)	
Inflammatory Bowel Diseases							
No	224 (95.3%)	118 (96.7%)	106 (93.8%)		108 (95.6%)	116 (95.1%)	
Familiarity	6 (2.5%)	2 (1.6%)	4 (3.5%)	0.57	3 (2.7%)	3 (2.5%)	0.99
Yes	5 (2.1%)	2 (1.6%)	3 (2.7%)		2 (1.8%)	3 (2.5%)	
Osteoporosis							
No	192 (81.7%)	94 (77.1%)	98 (86.7%)		84 (74.3%)	108 (88.5%)	
Familiarity	29 (12.3%)	20 (16.4%)	9 (7.9%)	0.12	18 (15.9%)	11 (9.0%)	0.06
Yes	14 (5.9%)	8 (6.6%)	6 (5.3%)		11 (9.7%)	3 (2.5%)	
Oral health status							
Periodontitis Staging¶							
Healthy/Gingivitis	34 (14.5%)	7 (5.7%)	27 (23.9%)		8 (7.1%)	26 (21.3%)	
Stage 1	27 (11.5%)	13 (10.7%)	14 (12.4%)		9 (7.9%)	18 (14.8%)	***0.03***
Stage 2	56 (23.8%)	27 (22.1%)	29 (25.7%)	***0.00***	25 (22.1%)	31 (25.4%)	
Stage 3	94 (40%)	58 (47.5%)	36 (31.9%)		56 (49.6%)	38 (31.2%)	
Stage 4	24 (10.2%)	17 (13.9%)	7 (6.2%)		15 (13.3%)	9 (7.4%)	
Periodontitis Grading¶							

No Periodontitis	34 (14.5%)	7 (2.9%)	27 (11.5%)		5 (14.5%)	29 (12.3%)	
A	21 (8.9%)	13 (5.5%)	8 (3.4%)	***0.00***	9 (3.8%)	12 (5.1%)	***0.02***
B	111 (47.2%)	65 (26.4%)	46 (19.6%)		60 (25.5%)	51 (21.7%)	
C	69 (29.4%)	37 (15.7%)	32 (13.6%)		39 (16.6%)	30 (12.8%)	
Number of teeth	24.2 [23.5, 24.9]	23.7±5.3	24.7±5.6	0.06	23.7±5.3	24.6±5.5	0.08
CAL	2.8 [2.7, 2.9]	2.9±1.1	2.4±1.1	***0.00***	2.9±1.0	2.4±1.1	***0.00***
PPD	2.5 [2.5, 2.6]	2.6±0.7	2.4±0.6	***0.02***	2.6±0.7	2.4±0.6	***0.03***
% PPD>4mm	9.3 [7.8, 10.8]	11.0±12.7	7.4±9.9	***0.00***	11.6±12.8	7.2±9.9	***0.00***
% PPD 5-6mm	7.7 [6.5, 8.9]	8.9±9.9	6.4±8.6	***0.00***	9.4±10.4	6.2±8.0	***0.00***
% PPD>6mm	4.0 [0.2, 8.2]	5.6±4.7	2.6±5.7	***0.00***	5.6±4.6	2.4±4.3	***0.00***
Furcation involvement, yes†	76 (32.3%)	42 (34.4%)	34 (30.1%)	0.28	46 (40.7%)	30 (24.6%)	***0.01***
Mobility, yes	91 (38.7%)	53 (43.4%)	38 (33.6%)	***0.04***	57 (50.4%)	34 (27.9%)	***0.00***
Number of bleeding pockets‡	7.3 [5.9, 8.7]	8.9±12.1	5.5±7.8	***0.00***	9.1±10.9	5.7±9.5	***0.00***
FMPS	50.6 [47.5, 53.7]	51.3±23.9	49.8±24.6	0.69	50.6±24.8	50.5±23.8	0.98
FMBS	28.5 [26.2, 30.8]	30.1±15.6	26.7±19.8	***0.02***	29.7±17.9	27.3±17.7	0.25
Teeth lost for periodontitis, yes	76 (32.3%)	51 (41.8%)	25 (22.1%)	***0.00***	49 (43.4%)	27 (22.1%)	***0.00***
Domiciliary plaque control							
Brushing frequency							
Not performed	4 (1.7%)	2 (1.6%)	2 (1.8%)		1 (0.9%)	3 (2.5%)	
Occasionally	46 (19.6%)	28 (22.9%)	18 (15.9%)	0.37	27 (23.9%)	19 (15.6%)	0.22
Every day	185 (78.7%)	92 (75.4%)	93 (82.3%)		85 (75.2%)	100 (81.9%)	
Toothbrush type, powered	122 (51.9%)	60 (49.2%)	62 (54.9%)	0.23	55 (48.7%)	67 (54.9%)	0.20
Interdental cleaning (IC)							
Not performed	75 (31.9%)	41 (33.6%)	35 (30.9%)		37 (32.7%)	38 (31.9%)	
Interdental floss	51 (21.7%)	27 (22.1%)	24 (21.2%)	0.78	25 (22.1%)	26 (21.3%)	0.98
Interproximal brushes	109 (45.9%)	54 (44.3%)	54 (47.8%)		51 (45.1%)	57 (46.7%)	
Frequency of IC							
Not performed	75 (31.9%)	42 (34.4%)	33 (29.2%)		37 (32.7%)	38 (31.2%)	
Occasionally	48 (20.4%)	26 (21.3%)	22 (19.5%)	0.56	23 (20.4%)	25 (20.5%)	0.99
Every day	112 (47.7%)	54 (44.3%)	58 (51.3%)		53 (46.9%)	59 (48.4%)	

Table.1 patients' characteristics by perceived stress and sleep quality level.

Note: results of continuous variables are reported as mean [95% Confidence Interval]; results of binary and categorical variables are expressed as number (proportion).

Abbreviations: BMI, body mass index; CAL, clinical attachment level; PPD, probing depth; FMPS, full-mouth plaque score; FMBS, full-mouth bleeding score; IC, interdental cleaning.

* $p<0.05$ is considered statistically significant.

† Class II/III furcation involvement according to the classification of Hamp *et al.* (1975).

‡ Defined as the number of sites with probing depth≥5mm and positive to bleeding on probing.

¶ According to the 2018 EFP/AAP classification.

Table 2. patients' characteristics summarized by the combination of perceived stress and sleep quality. Values with different superscript letters are different at the 5% level.

Variable	Low stress, good sleep quality n=71	Low stress, poor sleep quality n=42	High stress, good sleep quality n=51	High stress, poor sleep quality n=71	*p*-value*
Socio-demographic characteristics					
Age	50.2±16.5	54.7±15.5	51.0±14.7	59.2±10.3	0.05
BMI	25.2±4.7	26.4±6.4	24.9±4.1	25.7±4.2	0.66
Gender, females	38 (53.5%)	25 (59.5%)	26 (50.9%)	47 (66.2%)	0.19
Occupation					
Unemployed	11 (15.5%)	6 (14.3%)	13 (26.0%)	12 (16.9%)	
Employed	44 (61.9%)	25 (59.5%)	28 (56.0%)	36 (50.7%)	0.09
Retired	16 (22.5%)	11 (26.2%)	9 (18.0%)	23 (32.4%)	
Education					
Elementary/middle school	16 (22.8%)	9 (21.4%)	17 (33.3%)	27 (38.0%)	
High school	32 (45.7%)	20 (47.6%)	22 (43.1%)	33 (46.5%)	0.14
College or more	22 (31.4%)	13 (30.9%)	12 (23.5%)	11 (15.5%)	
Smoking					
Never	35 (49.3%)	19 (45.2%)	21 (41.2%)	32 (45.1%)	
Former	20 (28.2%)	10 (23.8%)	15 (29.4%)	22 (30.9%)	0.22
Smoker	16 (22.5%)	13 (30.9%)	15 (29.4%)	17 (23.9%)	
Familiarity for periodontitis, yes	24 (33.8%)	10 (23.8%)	20 (39.2%)	33 (46.5%)	0.19
Oral health status					
Periodontitis Grading†					
No Periodontitis	19 (26.8%)	5 (11.9%)	6 (11.8%)	4 (5.6%)	
A	5 (7.0%)	3 (7.1%)	7 (2.9%)	6 (2.6%)	
B	29 (40.9%)	20 (47.6%)	25 (10.6%)	37 (15.8%)	***0.02***
C	18 (25.4%)	14 (3.3%)	13 (5.5%)	24 (10.2%)	
Periodontitis Staging†					
Healthy/Gingivitis	21 (29.6%)	6 (14.3%)	5 (9.8%)	2 (2.8%)	
Stage 1	8 (11.3%)	6 (14.3%)	10 (19.6%)	3 (4.2%)	
Stage 2	21 (29.6%)	8 (19.1%)	10 (19.6%)	17 (23.9%)	***0.00***
Stage 3	17 (23.9%)	19 (45.2%)	21 (41.2%)	37 (52.1%)	
Stage 4	4 (5.6%)	3 (7.1%)	5 (9.8%)	12 (16.9%)	
Number of teeth	24.7±5.8	24.6±5.3	24.5±5.1	23.2±5.3	0.11
CAL (mm)	2.3±1.1[a]	2.6±1.1[a]	2.6±1.1[a]	3.1±0.9[b]	***0.00***
PD (mm)	2.4±0.6[a]	2.6±0.7[a]	2.6±0.6[a]	2.7±0.8[b]	***0.00***
% PD>4mm	5.7±7.9[a]	10.4±12.2[b]	9.3±11.9[b]	12.3±13.2[b]	***0.00***
% PD 5-6mm	4.9±6.7[a]	8.9±10.7[b]	7.9±9.3[b]	9.6±10.2[b]	***0.00***
% PD>6mm	2.9±5.0[a]	1.5±2.6[a]	2.2±6.5[a]	8.0±5.9[b]	***0.00***
Furcation involvement, yes‡					
Mobility, yes	17 (18.7%)[a]	21 (23.1%)[b]	17 (18.7%)[a]	36 (39.6%)[b]	***0.03***
Number of bleeding pockets§	4.5±7.2[a]	7.3±8.5[b]	7.4±11.9[b]	10.2±12.2[b]	***0.00***
FMPS	48.4±24.6	52.2±0.3	53.5±22.6	49.7±24.9	0.77
FMBS	24.3±18.2[a]	30.7±21.8[b]	31.5±16.1[b]	29.1±15.2[c]	***0.04***

Teeth lost for periodontitis, yes	11 (14.5%)[a]	14 (18.4%)[b]	16 (21.1%)[b]	35 (46.1%)[c]	***0.00***
Domiciliary plaque control					
Brushing frequency					
Not performed	1 (1.4%)	1 (2.4%)	2 (3.9%)	0 (0%)	
Occasionally	10 (14.1%)	8 (19.1%)	9 (17.7%)	19 (26.8%)	0.31
Every day	60 (84.6%)	33 (78.6%)	40 (78.4%)	52 (73.2%)	
Toothbrush type, powered	42 (59.2%)	20 (47.6%)	25 (49.0%)	35 (49.3%)	0.54
Interdental cleaning (IC)					
Not performed	22 (30.9%)	13 (30.9%)	17 (33.3%)	24 (33.8%)	
Interdental floss	16 (22.5%)	8 (19.1%)	10 (19.6%)	17 (23.9%)	0.91
Interproximal brushes	33 (46.5%)	21 (50.0%)	24 (47.1%)	30 (42.3%)	
Frequency of IC					
Not performed	21 (28.0%)	12 (16.0%)	17 (22.7%)	25 (33.3%)	
Occasionally	14 (29.2%)	8 (16.7%)	11 (22.9%)	15 (31.3%)	0.97
Every day	36 (32.1%)	22 (19.6%)	23 (20.5%)	31 (27.7%)	

Note: results of continuous variables are reported as mean [95% Confidence Interval]; results of binary and categorical variables are expressed as number (proportion).
Abbreviations: BMI, Body Mass Index; CAL, clinical attachment level; PD, probing depth; FMPS, full-mouth plaque score; FMBS, full-mouth bleeding score; IC, interdental cleaning.
[*] *p*-value of the Kruskal Wallis or Fisher's exact test for patients' characteristics across the four subgroups; $p<0.05$
[†] According to the 2018 EFP/AAP classification.
[‡] Class II/III furcation involvement according to the classification of Hamp *et al.* (1975).
[§] Defined as the number of sites with probing depth≥5mm and positive to bleeding on probing.

Table 3. Association between perceived stress, sleep quality and its components with Periodontitis.

Variable	ORs for Periodontitis							
	Crude ORs	95% CI		*p*-value*	Adjusted† ORs	95% CI		*p*-value*
		Lower	Upper			Lower	Upper	
Poor sleep quality	3.6	1.5	8.2	***0.00***	2.4	0.9	6.1	0.07
High perceived stress	5.2	2.2	12.4	***0.00***	4.8	1.8	12.9	***0.00***
Perceived stress and sleep quality								
Low stress, good sleep quality	REF.				REF.			
Low stress, poor sleep quality	2.5	0.9	6.9	0.07	2.2	0.7	6.7	0.19
High stress, good sleep quality	3.9	1.4	11.1	***0.01***	4.6	1.4	15.5	***0.01***
High stress, poor sleep quality	14.5	3.3	64.7	***0.00***	9.2	1.8	45.1	***0.00***
Components of sleep quality								
Subjective sleep quality								
Good	REF.				REF.			
Bad	2.9	0.7	12.8	0.21	2.2	0.4	10.9	0.35
Sleep latency								
Low	REF.				REF.			
High	1.5	0.4	5.2	0.55	1.2	0.3	5.3	0.78
Sleep duration								
At least 8 hours	REF.				REF.			
Less than 8 hours	1.1	0.5	2.2	0.85	0.9	0.4	2.1	0.82
Habitual sleep efficiency								
>85%	REF.				REF.			
<85%	3.7	1.0	12.6	***0.04***	3.4	0.9	13.2	0.08
Sleep disturbances								
Less than once a week	REF.				REF.			
At least once a week	1.6	0.6	3.9	0.34	0.9	0.3	2.8	0.87
Use of sleeping medications								
Less than once a week	REF.				REF.			
At least once a week	0.9	0.3	3.5	0.94	0.4	0.1	1.9	0.28
Daytime dysfunction								

Not at all/Occasionally	REF.				REF.			
Regularly	1.5	0.4	5.4	0.50	1.0	0.2	4.2	0.99

Abbreviations: ORs, odds ratios; CI, confidence interval; REF., reference category.
* $p<0.05$
†Adjusted for age, body mass index, gender, smoking, education and brushing frequency.

Table 4. Association between perceived stress, sleep quality and its components with Stage III/IV periodontitis.

Variable	ORs for Stage III/IV periodontitis							
	Crude ORs	95% CI		*p*-value*	Adjusted† ORs	95% CI		*p*-value*
		Lower	Upper			Lower	Upper	
Poor sleep quality	3.7	1.6	5.6	***0.00***	2.2	1.2	4.1	***0.01***
High perceived stress	2.6	1.5	4.4	***0.00***	2.5	1.3	4.6	***0.00***
Perceived stress and sleep quality								
Low stress, good sleep quality	REF.				REF.			
Low stress, poor sleep quality	2.6	1.2	5.8	***0.02***	2.6	1.0	6.5	***0.04***
High stress, good sleep quality	2.5	1.2	5.2	***0.01***	2.8	1.2	6.8	***0.02***
High stress, poor sleep quality	5.3	2.6	10.9	***0.00***	4.4	1.9	10.2	***0.00***
Components of sleep quality								
Subjective sleep quality								
Good	REF.				REF.			
Bad	2.6	1.2	5.7	***0.02***	1.9	0.8	4.8	0.15
Sleep latency								
Low	REF.				REF.			
High	3.4	1.4	8.4	***0.00***	3.1	1.1	8.7	***0.04***
Sleep duration								
At least 8 hours	REF.				REF.			
Less than 8 hours	3.5	1.9	5.5	***0.00***	2.4	1.1	3.6	***0.02***
Habitual sleep efficiency								
>85%	REF.				REF.			
<85%	1.1	0.6	1.9	0.2	1.00	0.5	2.0	0.99
Sleep disturbances								
Less than once a week	REF.				REF.			
At least once a week	2.1	1.1	3.9	***0.02***	1.2	0.6	2.5	0.64
Use of sleeping medications								
Less than once a week	REF.				REF.			
At least once a week	1.9	0.8	5.1	0.2	1.4	0.5	4.3	0.51

Daytime dysfunction								
Not at all/Occasionally	REF.				REF.			
Regularly	2.1	0.9	4.6	0.08	1.4	0.6	3.7	0.45

Abbreviations: ORs, odds ratios; CI, confidence interval; REF., reference category.
[*] $p<0.05$
[†]Adjusted for age, body mass index, gender, smoking, education and brushing frequency.

Table 5. Association between perceived stress, sleep quality and its components with Grade C periodontitis.

Variable	ORs for Stage III/IV periodontitis							
	Crude ORs	95% CI		*p*-value*	Adjusted† ORs	95% CI		*p*-value*
		Lower	Upper			Lower	Upper	
Poor sleep quality	1.5	1.0	2.7	***0.04***	1.4	1.0	2.8	***0.04***
High perceived stress	1.1	0.6	1.9	0.84	0.9	0.5	1.8	0.79
Perceived stress and sleep quality								
Low stress, good sleep quality	REF.				REF.			
Low stress, poor sleep quality	1.5	0.6	3.4	0.36	1.2	0.4	3.2	0.75
High stress, good sleep quality	0.9	0.4	2.1	0.82	0.7	0.3	1.8	0.75
High stress, poor sleep quality	1.5	0.7	3.1	0.27	1.2	0.5	3.0	0.63
Components of sleep quality								
Subjective sleep quality								
Good	REF.				REF.			
Bad	1.5	0.7	3.2	0.31	1.2	0.5	3.1	0.69
Sleep latency								
Low	REF.				REF.			
High	2.8	1.3	6.3	***0.03***	2.9	1.1	7.8	***0.03***
Sleep duration								
At least 8 hours	REF.				REF.			
Less than 8 hours	1.7	0.9	2.9	0.46	1.3	1.0	3.0	***0.04***
Habitual sleep efficiency								
>85%	REF.				REF.			
<85%	0.7	0.4	1.5	0.46	0.7	0.3	1.6	0.42
Sleep disturbances								
Less than once a week	REF.				REF.			
At least once a week	0.9	0.5	1.9	0.95	0.8	0.3	1.8	0.55
Use of sleeping medications								
Less than once a week	REF.				REF.			
At least once a week	1.4	0.5	3.6	0.53	0.9	0.3	3.0	0.89
Daytime dysfunction								

Not at all/Occasionally	REF.				REF.			
Regularly	2.2	1.1	4.9	***0.04***	1.4	0.5	3.6	0.48

Abbreviations: ORs, odds ratios; CI, confidence interval; REF., reference category.
[*] $p<0.05$
[†]Adjusted for age, body mass index, gender, smoking, education and brushing frequency.

7

7. Dental Caries Occurrence in Inflammatory Bowel Disease Patients: A Systematic Review and Meta-Analysis

Marruganti C, Discepoli N, Gaeta C, Franciosi G, Ferrari M, Grandini S. Dental Caries Occurrence in Inflammatory Bowel Disease Patients: A Systematic Review and Meta-Analysis.
Caries Res. 2021;55(5):485-495.

Introduction

Within the definition of inflammatory bowel diseases (IBD), 2 chronic, relapsing inflammatory processes of the gastrointestinal tract are encompassed: Crohn's disease (CD) and ulcerative colitis (UC). While inflammation in UC is characteristically described to involve both the colonic mucosal surface [Ordás et al., 2012] and the oral mucosa (in the form of aphthous stomatitis) [Dotson et al., 2010; Ordás et al., 2012] in CD, it retains a nonuniform transmural extension along any part of the gastrointestinal tract [Baumgart and Sandborn, 2012]. The current paradigm of IBD pathogenesis comprises a combination of aberrant immune response, genetic susceptibility, gut dysbiosis, and environmental factors such as smoking, antibiotics, oral contraceptives, and diet [Abraham and Cho, 2009; Chapman-Kiddell et al., 2010]. The Westernization of lifestyle and diet, with considerably higher intake of refined sugars, was appointed as a key factor in the escalating incidence of IBD across Europe and the rest of the world [Ooi et al., 2016].

The most frequent symptoms of IBD include abdominal pain, diarrhea, and rectal bleeding [Shivashankar and Lichtenstein, 2018]. Between 6% and 47% of patients were also reported to develop extra-intestinal manifestations of IBD, 25% of which occurred a median time of 5 months prior to IBD

diagnosis and 75% occurred afterward [Vavricka et al., 2015]. Extra-intestinal manifestations most frequently include arthropathies, skin diseases, cholangitis, and oral lesions such as aphthous ulcers and periodontitis. Indeed, a recent meta-analysis showed that the odds of periodontitis in IBD patients are 3-fold greater than those in IBD-free controls [She et al., 2020]. Some studies also reported an increased prevalence of dental caries in patients with IBD [Rooney, 1984; Brito et al., 2008; Ślebioda et al., 2011; Vavricka et al., 2013; Szymanska et al., 2014].

Many hypotheses were proposed to explain the biological plausibility of the link between IBD and caries. First of all, some studies reported an increased salivary composition of *Streptococcus mutans* and *Lactobacilli*spp. in IBD patients compared to IBD-free controls [Szymanska et al., 2014; Rodrigues et al., 2019]. This observation may be related to either the dietary changes during the active phases of IBD disease (i.e., increased sugar consumption) [Järnerot et al., 1983] and the gut dysbiosis with reduced microbial diversity typical of both CD and UC. Nonetheless, the association between caries and IBD has not been clearly elucidated yet. Confirmation of such association would pave the way for the promotion of activities targeted at the early detection and prevention of dental caries since the moment of IBD diagnosis. Moreover, the creation of behavioral interventions targeted to the common risk factors control [Sheiham and Watt, 2000] of IBD and dental caries could implement the long-term management of both diseases. Therefore, the current review aimed to systematically evaluate the occurrence of caries in patients with IBD compared to IBD-free controls.

Methods

Protocol and Registration

The following systematic review was performed according to the PRISMA statement [Page et al., 2021] and received the registration number from the Prospective Register of Systematic Reviews (CRD42020213441).

Eligibility Criteria

Studies investigating the presence of caries in patients with either CD or UC were included in the present review. The Population, Exposure, Control, Outcome, and Studies method was used to identify the eligibility criteria:

The exclusion criteria of studies were as follows: reviews or register study designs, lack of control group (healthy subjects), outcome measure (DMFT), or disease definition (either CD or UC).

Information Sources

The search strategy included the screening of electronic databases and hand searching in relevant journals and other sources, for example, references in reviews related to our research question. The screening and inclusion steps were reported according to the PRISMA flow diagram [Page et al., 2021].

Electronic Search

MEDLINE, Embase, Google Scholar, LILACS, and Cochrane Central Register of Controlled Trials electronic databases were screened. A search in grey literature (OpenGrey) was also carried out. The search strategy was built with a combination of MeSH terms and free-text words. Only articles in English were considered for the inclusion in the study, while no publication date restrictions were applied during the search. The full electronic strategy applied is reported for MEDLINE (through PubMed):

The search strategy was then modified for the other databases.

Hand Search

Hand search was performed independently by 2 calibrated investigators (C.M. and C.G.) in relevant journals (*International Endodontic Journal*, *Journal of Endodontics*, *Journal of Clinical Periodontology*, *Journal of Periodontology*, and *Inflammatory Bowel*

Diseases) evaluating issues published between January 1980 and April 2021. Other sources encompass narrative reviews and references of relevant articles.

Study Selection

Articles were first screened by title and abstract by 2 independent and calibrated reviewers (C.M. and C.G.) (unweighted Cohen's kappa score of 0.90). Whenever information was not clear at this stage, articles were selected for full-text analysis and reviewed by both investigators according to the inclusion and exclusion criteria. Any disagreement at this stage was resolved through discussion with a third author (S.G.).

Data Collection Process

Data collection was carried out through an extraction sheet by 2 independent reviewers (C.M. and C.G.) during full-text analysis. Study characteristics included: country, study design, matched variables, number of participants (proportions of males and females), age, disease definition, disease activity (IBD), and dietary habits (meal frequency). Authors were contacted whenever information was unclear at this stage.

Data Items

The primary outcomes of the present review protocol were the presence of caries in patients with IBD versus healthy subjects. The presence of caries was evaluated through the DMFT index [World Health Organization, 2013]. Weighted mean differences (WMDs) in DMFT values between IBD and control subjects were then computed.

Risk of Bias in Individual Studies

Overall quality of the included studies was assessed with the Newcastle-Ottawa Scale (NOS) for case-control studies [Wells et al., 2009].

It encompasses 3 categories:

1. selection of cases and controls (4 items);

2. comparability of cases and controls on the basis of the design or analysis (1 item);

3. exposure (3 items).

Each study was given a maximum of one star per each item of the Selection and Exposure categories and a maximum of 2 stars in the Comparability section. The overall quality for each study was therefore assessed on a scale of 0–9 points. Whenever a summary score ≥5 was reached, the study was rated as moderate or high quality. Cross-sectional studies were evaluated through the Critical Appraisal Checklist for Analytical Cross-sectional studies (Joanna Briggs Institute, University of Adelaide; http://joannabriggs.org/research/critical-appraisal-tools.html); it consists of 8 questions with 4 possible answers: yes ("low risk of bias"), no ("high risk of bias"), unclear ("unclear risk of bias"), and not applicable. Any disagreement between investigators at this stage was resolved through discussion; in case consensus was not reached, a third investigator (S.G.) was involved. Inter-examiners agreement was calculated using the kappa score.

Summary Measures

Data were pooled for both qualitative and quantitative analysis. The DMFT index was considered as the primary outcome measure and reported as mean and standard deviation. Whenever it was not present, an attempt was made to contact authors and obtain raw data. The estimate of the effect was calculated as the WMD for the DMFT index.

Risk of Bias across Studies

The overall quality of evidence at the outcome level (DMFT index) was evaluated using the Grades of Recommendation, Assessment, Development, and Evaluation approach [Guyatt et al., 2011]. The quality of evidence was

rated on a four-level scale (very low, low, moderate, and high) according to study design, risk of bias, inconsistency, indirectness, and imprecision; each of these items was given a judgment (very serious, serious, and not serious) [Guyatt et al., 2009a]. On these grounds, the strength of recommendation was deemed critical, important, or not important [Guyatt et al., 2009b].

Synthesis of Results and Additional Analyses

All analyses were performed using an ad hoc statistical software (version 16.1, STATA IC, Stata Corp) setting the level of significance at $\alpha = 0.05$. The Q test based on χ^2 statistics as well as the I^2 index were used in order to identify the percentage of variation in the global estimate attributable to heterogeneity. It was defined as: absent (I^2: 0–25%), low (I^2: 25.1–50%), moderate (I^2: 50.1–75%), or high (I^2: 75.1–100%). Due to the high heterogeneity across studies, the DerSimonian and Laird random-effect model [DerSimonian and Laird, 1986] was applied to conduct the meta-analysis using the means and standard deviations for DMFT values.

The DMFT index for each study was expressed as the WMD with 95% confidence interval (95% CI) between cases and controls, and then, a subgroup analysis was performed according to disease definition (CD or UC). Publication bias was assessed through the Egger's test and visualized as funnel plot. Additionally, the jack-knife sensitivity analysis omitted one study at a time and investigated whether one study had an exaggerated effect on the pooled estimates.

Results

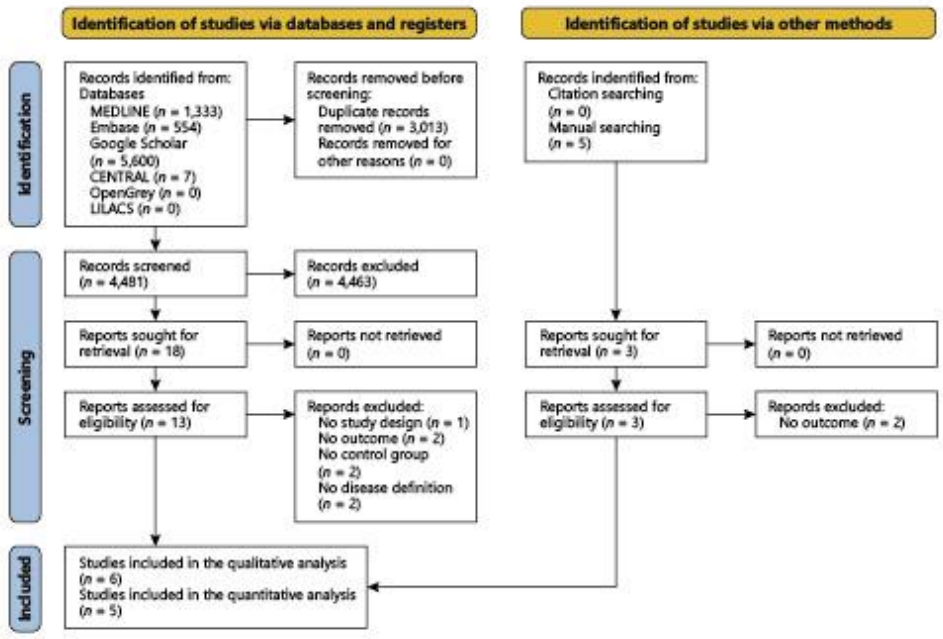
Study Selection

Initial electronic search retrieved 1,333 records in MEDLINE, 554 in Embase, 5,600 in Google Scholar, 7 in Cochrane Central Register of Controlled Trials, and 0 in OpenGrey and LILACS. Hand-search from

relevant journals led to 5 additional articles and none from other sources. After duplicates removal, 18 records were selected at the abstract level according to the inclusion and exclusion criteria. Thirteen full-text articles were assessed for eligibility, 6 of which were included in the qualitative analysis. All studies except for one [Zhang et al., 2020] were included in the quantitative (meta-analysis) synthesis of data. Reasons and timing of exclusion for each study are presented in online supplementary File 1 (see www.karger.com/doi/10.1159/000519170 for all online suppl. material). Investigators agreement for articles inclusion resulted in a k score of 0.90. A PRISMA flow diagram summarizing all inclusion stages is presented in Figure 1.

Fig. 1.

PRISMA flow diagram summarizing all inclusion stages.



Study Characteristics

Characteristics of the included studies [Brito et al., 2008; Ślebioda et al., 2011; Vavricka et al., 2013; Szymanska et al., 2014; Szczeklik et al., 2017; Zhang et al., 2020] are shown in Table 1. Two studies were conducted in

Poland [Ślebioda et al., 2011; Szczeklik et al., 2017], and the other 4 studies were conducted in Brazil, Sweden, Switzerland, and China, respectively, [Brito et al., 2008; Vavricka et al., 2013; Szymanska et al., 2014; Zhang et al., 2020]. One study [Zhang et al., 2020] had a cross-sectional design, while the others were case-control studies [Brito et al., 2008; Ślebioda et al., 2011; Vavricka et al., 2013; Szymanska et al., 2014; Szczeklik et al., 2017]. For the primary outcome (DMFT index), participants were a total of 997 IBD (age range: 29–46.1, proportion of males/females: 547/450) and 658 healthy subjects (age range: 26–48.6, proportion of males/females: 322/336).

Table 1.

Characteristics of the included studies

Author, year	Country	Study design	Matched variables	No. of participants (M/F)	Age, years, mean (SD)		Disease definition		Subjects with active disease (IBD)	Dietary habits (No. of meals/day)	NOS score
					IBD	control	IBD	caries			
Brito et al. [2008]	Brazil	Case-control	Age	IBD: 179 (64/115) CD: 99 (31/68) UC: 80 (33/47) Controls: 74 (24/50)	CD: 39 (12.9) UC: 43.3 (13.2)	40.3 (12.9)	Clinical, radiological, endoscopic and histological analysis	DMFT[a]	CD: 22 (22.2%) UC: 19 (23.7%)	nr	6
Ślebioda et al. [2011]	Poland	Case-control	None	IBD: 95 (53/42) CD: 70 (37/33) UC: 25 (16/9) Controls: 70 (24/46)	CD: 37.4, M: 37.4, F: 37.5 UC: 37.2, M: 39.8, F: 36.6	Controls: 31.6 M: 30.4 F: 33.5	nr	DMFT[a]	nr	nr	2
Szczeklik et al. [2017]	Poland	Case-control	Age, sex	CD: 71 (47/24) (urban/rural areas: 37/34) Controls: 61 (37/24) (urban/rural areas: 32/29)	CD: 33.4 (3.1) (urban/rural areas: 32.5 [3.5]/34.1 [3.2])	36.2 (2.8) (urban/rural areas: 35.2 [4.6]/37.2 [3.3])	nr	Number of DT, MT, FT, DMFT[a]	Mean (SD) CDAI score: 201.7 (15.1)	nr	6
Szymanska et al. [2014]	Sweden	Case-control	Age, sex	IBD: 150 (73/77) CD: no resective surgery 79 (40/39) resective surgery 71 (33/38) Controls: 75 (29/46)	CD: No resective surgery 42.0 (14.4) Resective surgery 50.7 (13.9)	48.6 (13.4)	Lennard Jones criteria	DMFT, DT, MT, FT[b]	nr	CD: no resective surgery 4.01 (1.4) Resective surgery 3.97 (1.5) Controls 4.06 (1.5) Consumption of sweetened drinks: CD: no resective surgery (34/43%) Resective surgery (43/61%) Controls (20/26%)	9
Vavricka et al. [2013]	Switzerland	Case-control	Age	IBD: 113 (65/48) CD: 69 (37/32) UC: 44 (28/16) Controls: 113 (58/55)	IBD: 40.6 (13.3) CD: 39.6 (12.1) UC: 42.3 (14.0)	41.7 (16.0)	Clinical, radiological, endoscopic and histological analysis	DMFT[a]	nr	nr	7
Zhang et al. [2020]	China	Cross-sectional	Age, sex	IBD: 389 CD: 265 (170/95) UC: 124 (75/49) Controls: 265 (150/115)	CD: 29 (25–36)[a] UC: 30 (27–40)[a]	26 (25–47)[a]	Third European evidence-based consensus on diagnosis and management of CD and UC criteria	DMFT, DT, FT, MT[b]	CD: 96 (36.2%) UC: 49 (39.5%)	CD (No. of subjects): ≤2 meals/day: 34 (12.8) 3 meals/day: 204 (77.0) 4 meals/day: 21 (7.9) 5 meals/day: 6 (2.3) UC (No. of subjects): ≤2 meals/day: 13 (10.5) 3 meals/day: 97 (78.2) 4 meals/day: 13 (10.5) 5 meals/day: 1 (0.8) Controls (No. of subjects): ≤2 meals/day: 33 (12.5) 3 meals/day: 214 (80.8) 4 meals/day: 14 (5.3) 5 meals/day: 4 (1.5)	Low risk of bias*

No, number; M, males; F, females; SD, standard deviation; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; DMFT, decayed missing filled teeth (permanent teeth); nr, not reported; CDAI, Crohn's disease activity Index; DT, decayed teeth; MT, missing teeth; FT, filled teeth; vs, versus; NOS, Newcastle-Ottawa Scale. [a]Outcome reported as the median (interquartile range). [b]Number of decayed, missing (for caries reasons), and filled teeth. *Critical Appraisal Checklist for Analytical Cross-sectional Studies, Joanna Briggs Institute, University of Adelaide.

Risk of Bias in Individual Studies

Inter-examiners agreement resulted in an unweighted Cohen's kappa score of 0.90. Individual and summary scores according to the NOS for each study are presented in online supplementary File 2. All studies, except for one [Ślebioda et al., 2011], resulted in a moderate to high methodological quality (summary score ≥5). In the Selection category, 4 out of 5 studies lacked the definition of controls (healthy subjects). On the other hand in the Comparability category, all studies except for one [Ślebioda et al., 2011] matched cases and controls for at least the most important factor (age); 3 studies out of 5 did not match patients for any additional factor (e.g., sex and socioeconomic status)[Brito et al., 2008; Ślebioda et al., 2011; Vavricka et al., 2013]. In the Exposure category, 3 studies out of 5 had different nonresponse rates for the 2 groups [Brito et al., 2008; Ślebioda et al., 2011; Szczeklik et al., 2017]. The risk of bias assessment of the cross-sectional study [Zhang et al., 2020] resulted in "low risk of bias" for all 8 items (online suppl. File 3).

Results of Individual Studies and Synthesis of Data

One study [Zhang et al., 2020] was excluded from the quantitative synthesis of data as the outcome measure could not be computed through raw data analysis with the available information. When comparing IBD to healthy controls, the WMD (95% CI) in the DMFT index was 3.04 (1.52, 4.56) (p = 0.00) (shown in Fig. 2). Subgroup meta-analysis according to disease definition (CD vs. UC) resulted in a WMD (95% CI) of 2.52 (0.54, 4.49) for CD and of 4.01 (1.92, 6.09) for UC (shown in Fig. 3). No statistically significant differences were found between the 2 subgroups (p = 0.31). No publication bias was detected (p = 0.19); visual inspection of the funnel plot confirmed the presence of a symmetrical distribution of studies (online suppl. File 4). Results of the jack-knife sensitivity analysis (online suppl. File 5) did not significantly influence effect estimates, which ranged between 1.16 and 5.10 and remained statistically significant.

Fig. 2.

DMFT levels (WMD 95% CI) in IBD versus healthy controls (Random-effects DerSimonian-Laird model). DMFT, Decayed, Missing, Filled Teeth; WMD, weighted mean difference; CI, confidence interval; IBD, inflammatory bowel disease.

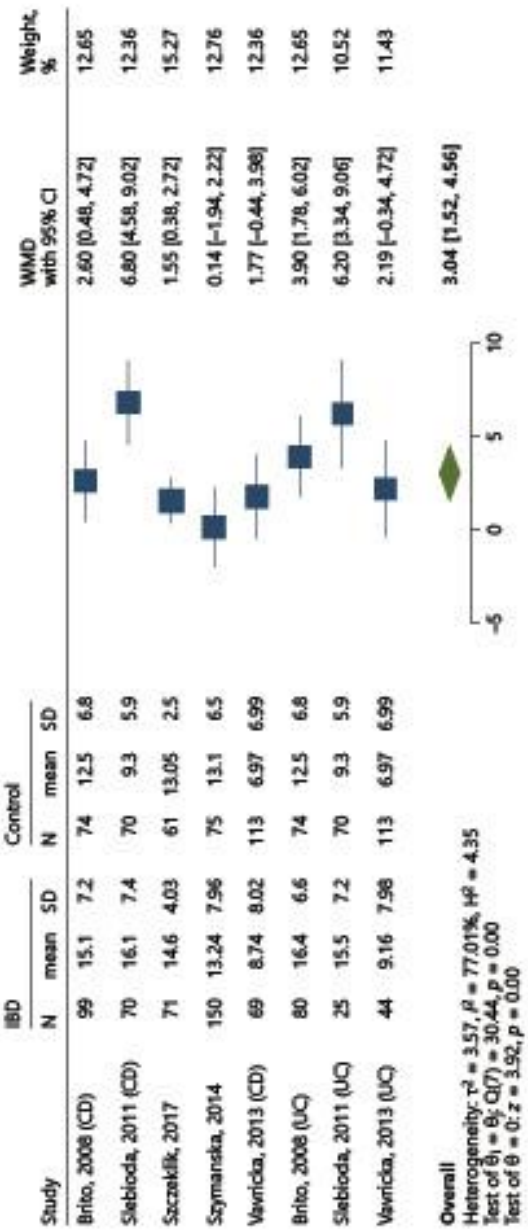
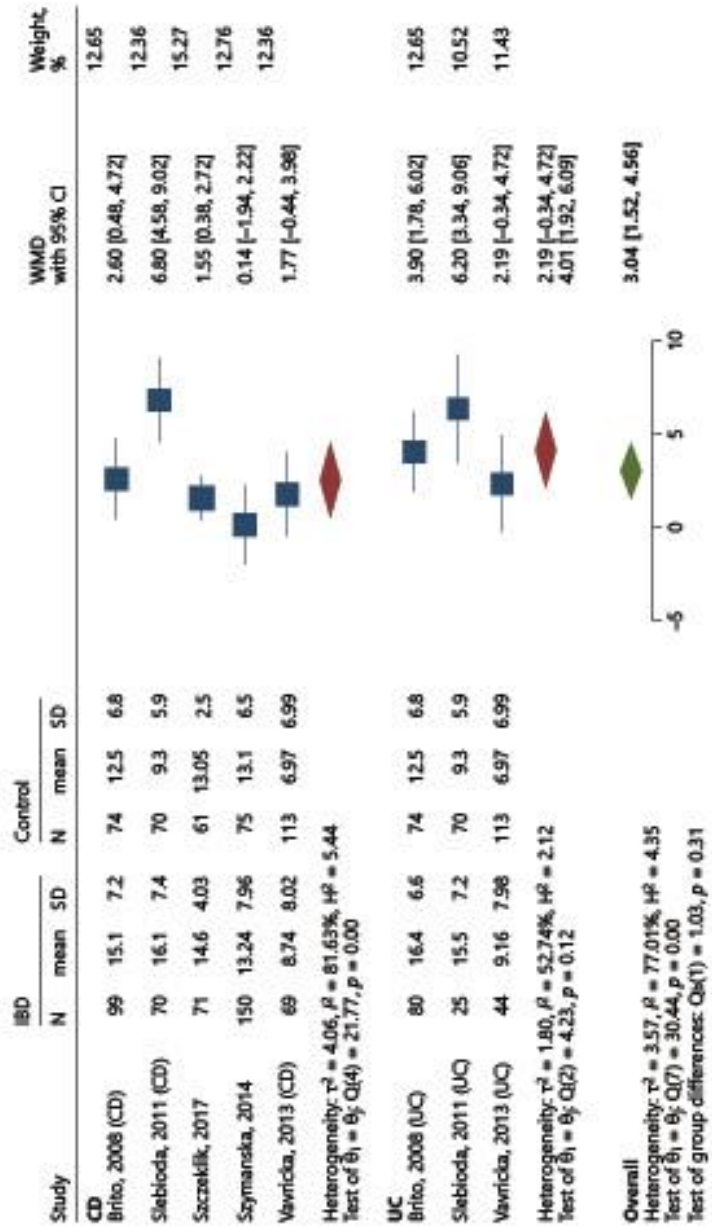


Fig. 3.

Subgroup analysis according to disease definition (CD vs. UC) (Random-effects DerSimonian-Laird model). CD, Crohn's disease; UC, ulcerative colitis; WMD, weighted mean difference; CI, confidence interval; IBD, inflammatory bowel disease.



Risk of Bias across Studies

Results of the Grades of Recommendation, Assessment, Development, and Evaluation are presented in Table 2. All quality assessments started from a "low" level due to the inclusion of observational studies [Guyatt et al., 2009a]. The quality rating was rated up by one level due to the large effect included in the CI [Guyatt et al., 2009b]. Strength of recommendation was rated as "critical" for the DMFT index.

Table 2.

Quality assessment using the GRADE approach

Quality assessment								Summary of findings			Importance
								Patients, n		effect size (WMD)	quality
Study groups, n	study design	risk of bias	inconsistency	indirectness	imprecision	other considerations		IBD	healthy		
Population: human subjects Settings: University clinics/hospitals (Brazil, Poland, Sweden, and Switzerland) Exposure: diagnosis of any IBD (either CD or UC) Comparison: IBD-free subjects Outcome, missing, filled teeth (DMFT)											
8	Case-control	Not serious	Not serious	Not serious	Not serious	None		608	393	3.04 (1.52, 4.56)	Moderate* ⊕⊕⊕◯	CRITICAL

All quality assessments started from a "low" level due to the inclusion of observational studies. IBD, inflammatory bowel disease; WMD, weighted mean difference; OR, odds ratio; CD, Crohn's disease; UC, ulcerative colitis; GRADE, grading of recommendations assessment development and evaluation; CI, confidence interval. *Raised up by one level due to the large effect included in the CI.

Discussion/Conclusion

Summary of Evidence

The current systematic review focuses on the population-based index of caries experience (DMFT) in patients with IBD compared to healthy controls. Overall, results showed significantly higher values of DMFT index in IBD patients versus healthy controls ($p < 0.05$); subgroup meta-analysis demonstrated no differences in the oral health status of CD and UC patients. The increased caries experience in CD and UC patients compared to systemically healthy subjects could be attributed to various reasons. First of all, increased meal frequency as well as higher sugar and carbohydrates intakes were reported for both CD and UC patients [Järnerot et al., 1983; Schütz et al., 2003; Grössner-Schreiber et al., 2006; Szymanska et al., 2014; Głąbska et al., 2019]. In particular, patients during the active phases of CD tend to eat sugary foods more frequently as they plausibly ease the gastrointestinal symptoms associated with disease exacerbation [Järnerot et al., 1983]. Significantly higher daily sugar intakes were recently demonstrated also for patients with UC in remission versus non-IBD controls (mean: 20 g/day and 10.6 g/day, respectively) [Głąbska et al., 2019]. Only 2 of the included studies reported dietary habits registered through a questionnaire [Szymanska et al., 2014; Zhang et al., 2020]. Both studies did not highlight significant differences as to meal frequency; nonetheless, Szymanska and coworkers [Szymanska et al., 2014] demonstrated an increased consumption of sweetened drinks between meals in CD compared to non-IBD patients. Sugar consumption and meal frequency are undoubtedly related to caries risk [Sheiham and James, 2015]; indeed, they retain a huge variability across different countries and regions. The highest DMFT values in the present systematic review were registered in studies conducted in Eastern Europe [Ślebioda et al., 2011; Szczeklik et al., 2017]. It can be hypothesized that sugar intake played a pivotal role in this observation. In fact, the findings of a recent population-based cohort study from the European Crohn's and Colitis Organization (ECCO) Epidemiological Committee (EpiCom), demonstrated a significantly higher sugar intake in IBD patients from Eastern Europe than in those from Western Europe [Burisch et al., 2014]. Nonetheless, sugar consumption was not registered in neither of the 2 studies conducted in Eastern Europe [Ślebioda et al., 2011; Szczeklik et al., 2017]. Additionally, changes in the salivary composition were also found to

play a key role in the higher caries experience detected in IBD patients [Szymanska et al., 2014; Rodrigues et al., 2019]. Only one of the included studies analyzed the salivary microbiological composition in CD versus healthy subjects [Szymanska et al., 2014], thus demonstrating higher concentrations of *Streptococcus mutans* and *Lactobacilli*spp. in the saliva of CD patients versus controls. This is consistent with previous reports stating that while on one hand no variations of salivary flow rate and buffering capacity are present [Sundh and Emilson, 1989], on the other hand modifications in the concentration of acidogenic bacteria take place in both CD [Sundh and Emilson, 1989] and UC [Rodrigues et al., 2019] patients. However, evidence with this regard is still conflicting [Halme et al., 1993; Meurman et al., 1994]. Szymanska and coworkers [Szymanska et al., 2014] also highlighted a significant difference in caries experience, sweetened drinks consumption, and salivary counts of cariogenic bacteria between CD patients undergoing resective surgery and those who did not; hence, a relationship between the severity of the disease (leading to the need for surgery), a higher sugar intake, and thus an increased caries experience is suggested.

Part of the observed heterogeneity could also have been related to disease activity; as previously stated, IBD patients were reported to increase sugar consumption especially during the active phases of the disease [Järnerot et al., 1983]. Only 2 of the included studies recorded this information; in particular, Brito and coworkers [Brito et al., 2008] reported that almost half of the cases had active disease; moreover, Szczeklik and coworkers [Szczeklik et al., 2017] recorded a mean CD Activity Index score which is consistent with mildly active IBD [Jørgensen et al., 2005]. Indeed, DMFT values reported by both studies were among the highest for both CD and UC; therefore, a further relationship between disease activity and caries occurrence together with the mediating role of sugar intake can be hypothesized.

The present systematic review highlighted a burdensome past and present caries experience as well as an urgent need for dental treatments in patients with IBD; these findings should encourage health-care providers and policy

makers to create oral health-care programs targeted at a high-risk stratum of society like subjects diagnosed with IBD. In particular, these programs should entail behavioral interventions for risk factors control (oral hygiene habits, sugar consumption, and meal frequency), as well as a prompt intervention to prevent the onset of the disease (primary prevention) or to treat the disease while at the early stages (secondary prevention) in order to avoid its clinical sequelae (e.g., apical periodontitis and tooth loss).

Level of Evidence

The body of evidence in individual studies was of moderate to high quality overall. One study only resulted in low quality [Ślebioda et al., 2011], while all the other studies achieved a NOS summary score consistent with moderate or high methodological quality. The most frequently violated item was the definition of controls; in fact, many of the included studies lacked an accurate description of the comparison group, thus leaving to question whether control subjects were exposed to the same risk factors and confounders as the cases and whether they were representative of the population that constituted the cases. Nonetheless, the efficiency of the adjustment for confounders was increased by matching [Stang, 2010]; indeed, all studies except for one [Ślebioda et al., 2011] matched for at least the most important factor (age). On the other hand, around half of the included studies had different numbers of IBD and non-IBD respondents; the different nonresponse rate was addressed by none of the studies, except for one [Szymanska et al., 2014], hence posing the risk for an overestimation of the effect of the exposure on the outcome.

Despite all ratings started from "low level" because of the observational nature of the included studies [Guyatt et al., 2009a], the overall assessment resulted in "moderate quality" due to the large effect included in the CI [Guyatt et al., 2009b]. All in all, the strength of recommendation as to the association between dental caries and IBD was judged as "critical." Therefore, these results should serve as a call to action for clinicians to guide

IBD patients into structured oral health prevention and intervention programs from the moment of IBD diagnosis.

Strengths and Limitations

The strengths of the present review encompass the strict methodology used during every step according to the most recent guidelines [Page et al., 2021] as well as the fairly high number of included subjects (total of 1,655 subjects). Moreover, these findings constitute the first metadata so far regarding caries experience in CD and UC patients separately. However, it presents some inherent limitations. First of all, a limited number of studies was included and therefore heterogeneity, whenever present, could not be fully addressed. In fact, a high amount of heterogeneity may be due to some inherent characteristics of the outcome variable (DMFT). Indeed, DMFT is a continuous-discrete variable ranging between 0 and 28; its values tend to be skewed to the right (positively skewed) and with an excess of zeros (with a possible thicker tail); as such, DMFT is seldom normally distributed. Moreover, part of the heterogeneity could be pointed out to the operator-dependency of the outcome measurement (DMFT) [World Health Organization, 2013] as well as other genetic and environmental factors influencing caries pathogenesis [Selwitz et al., 2007] that could not be accounted for in the meta-analysis. Furthermore, despite the pivotal role of diet [Sheiham and James 2015] and salivary characteristics (i.e., salivary flow rate, buffering capacity, and count of cariogenic bacteria) [Gao et al., 2016] on caries experience, only few of the included studies reported these data; therefore, their mediating role between dental caries and IBD could not be thoroughly analyzed. Indeed, given the paucity of the included studies, the presence of publication bias could not be completely ruled out. Finally, another concern regards the impossibility to infer neither causality nor temporality between caries and IBD due to the lack of longitudinal studies.

Conclusion

Our study reveals a remarkably higher past and present occurrence of dental caries in patients with IBD, either CD or UC, when compared to healthy controls. Therefore, we propose that oral health programs, which should

include the early detection of dental caries and behavioral interventions for risk factors modification (mainly sugar consumption), be encompassed in the treatment plan of IBD patients from the moment of diagnosis. Further studies are needed to evaluate the presence of a cause-effect relationship between dental caries and IBD.

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8. Evaluation of two endodontic irrigation needles on curved root canal disinfection: an ex vivo study

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1. Introduction

Enterococcus faecalis is a Gram-positive coccus which is frequently isolated from secondary endodontic infections [1]. *E. faecalis* is capable to survive despite prolonged periods of nutrient deficiency [2], and it was demonstrated to be resistant to endodontic antimicrobials during endodontic treatment [3]. This is related to the ability of *E. faecalis* to form dense biofilms and to invade dentinal tubules and root canal complexities [3, 4], thus surviving the antibacterial action of sodium hypochlorite and better resisting its flushing action during injection into the canal space [5]. Even though sodium hypochlorite is capable to quickly oxide the membranes of those bacteria located more superficially within biofilm, it is not so effective on deeply embedded bacteria that remain protected from the direct action of chlorine [5].

To obtain a successful treatment outcome, microorganism should be eliminated from the root canal system [6] or at least reduced as much as possible in order to be compatible with healing [7].

Although microorganisms can be eliminated from the endodontium by mechanical instrumentation, some persist, especially in the presence of isthmuses, deltas, lateral canals, ramifications and complex anatomies [5]; in particular, complete microbial eradication from the apical third of the canal seems to be the most difficult goal to pursue, since 10-50% of the walls are

not touched by root canal instruments [8], regardless of the instrumentation technique employed [9].

Moreover, mechanical tools tend to produce a substantial amount of smear layer and dential debris and to push them into tubules and irregularities, creating an organo-mineral content layer where microorganisms hide from irrigants and intracanal medication to survive and multiply.

Microorganisms escaping chemo-mechanical preparation are currently considered the leading cause of failure after primary treatment and non-surgical retreatment [10]. Endodontic irrigation is essential for root canal debridement, representing the only mean available to clean areas that cannot be reached through mechanical instrumentation[11]. To achieve complete eradication of *E. faecalis* from the endodontic system, penetration of the irrigating solutions inside dentinal tubules is necessary and can be achieved by smear layer removal from canal walls[12].

Clinically, irrigation with a syringe and a needle remains the most used procedure to inject the solution deep into the root canal [13], even though its efficacy in the apical part is uncertain [12]. Current evidence suggests that size, design and insertion depth of the irrigation needle tip are of primary importance in the elimination of bacterial biofilms [14,15]. According to the literature irrigation needles can be classified in two categories: *open-ended* needles that eject the irrigant directly through their tip and *closed-vented* needles with a closed tip ejecting the irrigant through one or more side vents [10].

NaviTip (Ultradent, South Jordan, UT, USA) is a stainless-steel open-ended cannula, slightly stiff at the base and center, but flexible at the end to allow easy penetration into curved canals [16,17].

IrriFlex (Produits Dentaires SA, Vevey, Switzerland) has been proposed as an alternative to traditional metal needles, which is a unique irrigation needle consisting of a double-side-vented soft polypropylene needle, adapting easily to the anatomy of root canals, even to the most complex ones. The flexibility of the polypropylene cannula allows apical access without resistance or damage to the dentinal walls, reaching the predetermined working length and avoiding any risk of extrusion in the periapical area; this

unique feature allows a balanced irrigant expulsion through two precise jets oriented directly against the dentinal walls [5,18].

The aim of the current study was to compare the effectiveness of removing *E. faecalis* from in vitro contaminated root canals by IrriFlex and Navitip irrigation needles. The null hypothesis was that there are no statistically significant differences between the two irrigation systems tested in the eradication of *E. faecalis* from the root canal.

2. Materials and Methods

2.1. Sample Collection and Preparation

This ex vivo study was approved by the ethical committee of Azienda-Ospedaliero Universitaria Senese number 7/2021. Thirty extracted multirooted teeth or both orthodontic and periodontal reasons, were collected and stored in phosphate buffered saline (PBS) until use. Each tooth was subjected to a radiological examination to assess the presence of walkable curved canals. A preliminary bidimensional radiographic examination was performed both in bucco-lingual and mesio-distal directions to ensure a comparable anatomy among teeth. Only those with an angle of curvature ranging from 25° to 40° degrees according to Pruett et al. evaluation method were selected [19]. Any teeth with decay or fractures below the cemento-enamel junction, internal or external resorption, open apices, or previous root canal therapy were excluded. The teeth surface was scraped to clean the soft tissue residues and disinfected by 5% sodium hypochlorite solution for 20 min. The cuspids of all teeth were removed until a uniform plane was established to promote a repeatable working length (WL). For access cavity preparation, a diamond bur mounted on a high-speed handpiece, equipped with a water-cooling system, was used. Access was performed following the design of the traditional access cavities to locate all the canal orifices. Then, WL of each canal was established with a size 10 K-File (Dentsply Maillefer, Ballaigues, Switzerland). The canals were then prepared with reciproc R25 files (VDW, Dentsply Maillefer, Ballaigues, Switzerland) to the full WL using a 6: 1 reduction handpiece (Sirona Dental Systems GmbH, Bensheim, Germany) connected to an electric motor (X-Smart Plus, Dentsply Maillefer) using the Reciproc ALL program (300 rpm, 150° REV–30° FWD) with an

up-and-down pecking motion as suggested by the manufacturer. Each instrument was used to prepare only one canal.

In order to ensure a perfect apical seal, the apices of the prepared roots of the teeth were sealed using flowable composite resin (Filtek Flow, 3 M-ESPE, St-Paul, MN, USA). At the same time, the orifices of the untreated canals were sealed with flowable resin composite. Subsequently, each tooth was placed in a stub created with putty-consistency silicone impression material (Zhermack, Badia Polesine, Veneto, Italy) to maintain it vertically in a glass jar. Samples were submerged in 10 mL of PBS and autoclaved at 121 °C for 25 min. The teeth were randomly divided in three groups: A (Irriflex group), B (Navitip group), C (Control group).

2.2. Laboratory Assessment

An *E. faecalis* strain (BE18), resistant to chemo-mechanical root canal preparation, was cultured anaerobically at 37°C on Fastidious Anaerobe Agar (LabM, Bury, UK) supplemented with 5% defibrinated horse blood. Starter cultures were set up in filter-sterilized modified fluid universal medium (mFUM), which were incubated anaerobically at 37°C for 3h, until the growth appeared moderately turbid. The turbidity was adjusted with fresh mFUM to an optical density of 0.5 at 540nm (Lab systems iEMS Reader MF, Basingstoke, UK). Preliminary studies had shown that after 72 h the biofilm was uniformly present over the surface of the root canal, so 20 ml of the washed *E. faecalis* culture will be pipetted into pretreated canals. Additionally, 1 ml of distilled water will be added to the base of the jar to provide a moist environment for both tooth and culture. The samples were then incubated at 37°C for 48 h.

2.3. Root Canal Contamination

Each root canal was contaminated with 10 µL of the bacterial suspension by inserting sampler microtip (Eppendorf, Hamburg, Germany) into the access cavities. Then, the bacterial suspension was introduced into the whole length of the canal using a #20 K-file up to the WL with a gentle pumping motion. If the turbidity of the sample medium reached 12×108 CFU/mL (equal to 4 McFarland) during the incubation period for all the samples, the

positive bacterial growth was confirmed. The specimens were stored in an incubator at 37°C and 95% relative humidity for 21 days. The purity of *E. faecalis* was identified by gram staining and microscopic observation of the colony morphology. The teeth were randomly divided into three groups (10 specimens in each experimental group). The initial microbial assessment (S1) was carried out after the incubation period using three sterile #25 paper points (Gapadent Co Ltd, Tianjin, China) that were kept in each canal for 1 min. In short, each root canal was filled with 20 µL sterile saline solution and a #20 K-file (Dentsply Maillefer, Ballaigues, Svizzera) was inserted into the root canals to reach the WL with a gentle filling motion. To detach the microorganisms from the inner root surfaces, an ultrasonic tip was placed in contact with the file shank and activated for 1 min; then, the root canals were sampled. Bacteria were recovered from the root canal using a series of five sterile paper points (size ISO 25, Dentsply), which were in turn rubbed against the walls of the root canals. The paper points were allowed to draw up their full capacity of liquid before to be transferred into a dry tube containing BHI media under aseptic conditions and frosted immediately at -20 C. After this stage, the contaminated vials were exchanged with the empty sterile ones. Irrigation with 5 ml of 5.25% sodium hypochlorite solution at room temperature was performed in the group A with IrriFlex and in the group B with NaviTip until the WL was achieved. The irrigant exposure time for both groups A and B was 30 seconds. The procedure was performed using digital pressure with the forefinger only, and the needle was gently moved back and forth in the canal ensuring that the needle did not bind in the canal itself. At the end of irrigation, the canals were rinsed copiously with sterile saline solution to flush away residual irrigants. After completing chemical preparation, the samples in groups A and B were irrigated with 5 mL of 5% sodium thiosulfate as a neutralizer and then rinsed with 5 mL sterile saline. Final sampling (S2) was performed using a similar method for initial sampling. *E. faecalis* colony forming units were enumerated by performing serial dilutions and growth on nutrient agar (NA; Oxoid, Basingstoke, Hampshire, UK). After 48 h aerobic incubation at 37°C. The same cohort of teeth was used throughout, and all irrigation experiments were conducted in triplicate.

2.4. Statistical analysis

The sample size was determined based on the data of a previous study regarding the NaviTip ability to eradicate E. Faecalis from root canal system [20]. A total of 24 samples were indicated as the ideal size required for noting significant differences using G∗Power v3.1 (Heinrich Heine, University of Düsseldorf, Düsseldorf, Germany) by selecting the analysis of variance (ANOVA) test and setting an alpha-type error of 0.05, a beta power of 0.90, and an effect size of 0.80. However, an additional 11 samples (5 for group A and B and 1 sample as control) were added in order to compensate for unexpected values of IrriFlex because there were no data in the literature regarding its chemio-mechanical properties. According to this, a total of 35 samples were selected.

The normality of the results was analyzed by both visually and Shapiro-wilk test. Differences between the groups were then analyzed by Kruskal-Wallis's test. Dunn tests were used for pairwise comparisons among the groups. Significance level was set to $p < 0.05$.

3. Results

A mean of 7.8 x 10 ^ 4 CFUs was reported for control samples, while the use of NaviTip and IrriFlex needles has afforded a reduction in bacterial viability below the positivity threshold set at 2.5 CFUs (Figure 1) without a statistical difference between the two irrigation needles ($p > 0.05$) (Table 1). Bacterial viability was significantly reduced after sodium hypochlorite irrigation using NaviTip and IrriFlex compared to controls. The mean log of 17.642 reported for untreated teeth turned out to be much higher compared to both test groups as shown in Table 2.

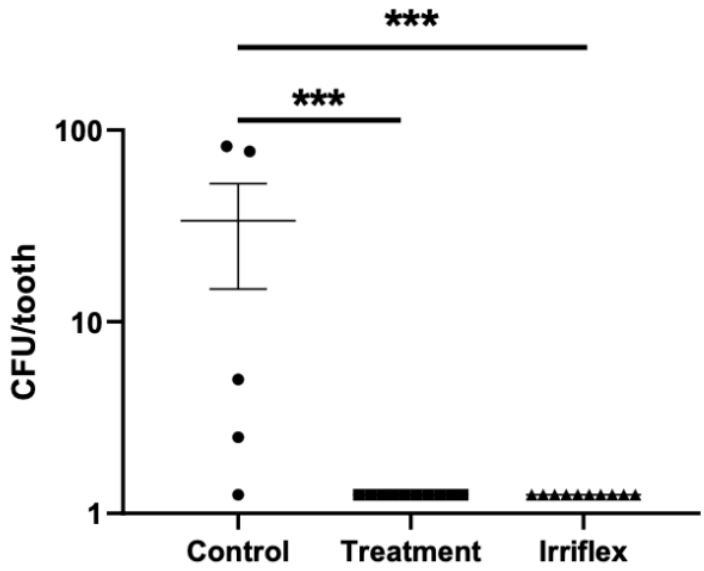

Figure 1. Mean count in CFUs. Bacterial viability after passive irrigation using NaviTip and Irriflex compared to controls.

Table 1. Statistical comparison between two different types of cannulas (Dunn test) in an extracted tooth model with curved canals for endodontic irrigation.

Cannule		Significant Differences (A-B)	P Value	Confidence Interval (95%)	
A	B			Minimum Value	Maximum Value
IrriFlex	Control	15.312	0.3891	59.354	28.731
NaviTip	Control	15.312	0.3891	-59.354	28.731
IrriFlex	NaviTip	0	0	0	0

Table 2. Mean log CFU of *E. faecalis* in curved canals of multirooted teeth after treatment in the test group.

Cannule	Bacteria Recovered (mean log CFU)	n	Standard Deviation (SD)	Variance
Control group	17.642	5	34.94	1220.87
IrriFlex	1.25	15	-	-
NaviTip	1.25	15	-	-

4. Discussion

The aim of present study was to evaluate the influence of two different endodontic irrigation needles on the elimination of *E. faecalis* from curved canals of multirooted teeth contaminated in vitro with *E. faecalis*. The two needles tested were respectively flexible double side-vented polypropylene needles (IrriFlex) and open-ended steel needles (NaviTip). The null hypothesis was accepted, as no statistically significant differences emerged between the two groups tested.

Infection control relies mainly on irrigation system to achieve reduction in microbial agents [21]. Therefore, for an effective root canal irrigation, it is important that the irrigant gains direct contact with the entire canal wall surface, especially in the apical region [22]. To fulfill this requirement an effective delivery system is necessary [16]. In the current study both NaviTip and IrriFlex system demonstrated a comparable capacity to achieve significant bacterial reduction below the positivity threshold (Figure 1).

These results are in accordance with a recent study conducted on single-rooted teeth which demonstrated that both NaviTip and IrriFlex are more efficient at removing mature bacterial biofilms when compared to another needle, with a slight but not significant advantage for the IrriFlex needle [5]. This small discrepancy in results is probably due to some methodological differences such as the choice of a different *E. fecalis* strains and viability

assessment confirming that IrriFlex and Navitip do no have any statistically significant differences in removing E. faecalis from endodontic systems.

Current literature suggests that the extrusion of the irrigant near the working length results in a more efficient irrigation [8]. Undoubtedly, soft plastic needles are able to progress smoothly inside the root canals following the root anatomy, preventing wedge formation, especially in curved canals, ensuring a more effective irrigation in the apical region compared to metal needles [5]. Moreover, some authors have previously compared the effectiveness of lateral ejection needles with single ejection needles, demonstrating better results in terms of removal of debris and bacteria in those with lateral ejection of the irrigant [23]. Despite the evidence regarding debris removal, no other studies are present in the literature comparing the effectiveness of the two delivery systems on bacterial viability reduction in curved canals of multirooted teeth.

E. faecalis mature biofilms are frequently used as a model of endodontic infection for ex vivo studies [5]. Particularly, it has been employed in several previous studies for the evaluation of endodontic irrigants efficacy [24]. This Gram-positive coccus can proliferate without synergistic support from other bacteria and survive under long periods of nutrient deficiency, remaining viable after mechanical and chemical root canal preparation due to its capacity to form biofilm and to penetrate deep into dentinal tubules [2]. Recent studies have questioned its actual role in post-treatment apical periodontitis [25,26], concluding *that it* is often absent and, when detected, it is not among the most prevalent species [1,27,28]. Therefore, the interesting feature of *E. faecalis* is the ability to sustain a wide range of growth conditions, ensuring simple laboratory handling. Despite the several *E. faecalis* strains used in literature, in the present study the clinical strain BE18 was employed because resistant to chemo-mechanical preparation.

The exposure time of the irrigant used in this study appears to be shorter than the one currently used in clinical practice. Although, Dunavant et al. showed that the amount of *E. faecalis* elimination does not seem to be affected by an exposure time ranging from 1 to 5 minutes [17].

Regarding NaOCl concentrations, no agreement has been achieved yet and values ranging from 0.5 to 8.25% are commonly used for irrigation

during root canal preparation [29–32]. According to laboratory studies, the desirable effects of NaOCl seems to be a function of its concentration [32–36] while, recently, a systematic review demonstrated, with weak evidence, that higher concentrations may ensure an advantage [37]. Nevertheless, according to recent clinical studies, a significant difference in the antimicrobial effect among different NaOCl concentrations have not been detected [38,39]. Particularly, Verma et al. found no difference in healing after endodontic treatment when using 1% or 5.25% sodium hypochlorite for root canal irrigation.

In the current study, 5.25% sodium hypochlorite solution was used because it was demonstrated that higher concentrations of NaOCl reduce the time required to lower viable counts below the limit of detection [44]. *E. Faecalis* was significantly more resistant to NaOCl (0.5, 1.0, 2.5 and 5.25%) when compared with the other species tested (*Actinomyces naeslundii*, *Candida albicans*) [40].

The primary outcome in endodontics is the prevention or healing of apical periodontitis [41], scarce evidences on root canal irrigation were actually reported in the literature [42]. The reduction of the intracanal microbial load is certainly the most relevant surrogate end-point to study irrigants and irrigation systems [24] due to the critical role of bacteria in the development of pulpal and periapical diseases [43]. In single-rooted teeth this end-point is linked to the healing of apical periodontitis, nevertheless there is a need to prove these findings also in posterior teeth [24].

The extracted tooth model, already used in previous studies to compare different irrigation systems and different irrigation solutions ,allows easier and more effective control of the contamination of the endodontic system [44]. Of course, clinical studies compared to ex vivo studies enable to test irrigants under real environmental factors, representing a higher level of evidence [45]. However, many of these parameters cannot be controlled showing a large variation from one tooth to another, acting as potential confounders [46]. It is obvious that the efficiency of new irrigation systems or irrigants should not immediately be tested by *in vivo* studies. Instead, *in vitro* and *ex vivo* studies with rigorous control of confounders should be performed in order to select the appropriate candidates for *in vivo* studies [46].

The insertion depth of the needle tip should require standardization as well [47–50] as it has been defined in some previous studies on the binding point of these components inside the root canal [51,52]. Nonetheless, since this point is extremely variable, it may differ even in root canals with same apical size and taper. Hence, it is advisable to use the apical end of instrumentation as a reference to define the insertion depth. Moreover, the constant in-and-out movement of these components along the root canal, which may be applied by some clinicians, is difficult to standardize in laboratory studies without appealing to robotic arms.

The study has potential limitations. Paper points can be employed for sampling when the biofilm is grown inside a root canal [53–56], however presenting some weaknesses[57]. Indeed, paper points are only capable of detaching planktonic bacteria from the root canal lumen and those bacteria that are only loosely adherent to the wall. Therefore, this sampling procedure excludes all the bacteria remaining in isthmuses, lateral canals and other anatomic irregularities that are also difficult to reach with instruments and irrigants [57]. Furthermore, the vortexing movement used to recover the sampled bacteria with the paper point causes loss of information on the exact localization of the bacteria in the root canal [24]. Moreover, the bacterial load in the sampled areas may not represent bacteria left in isthmuses, lateral canals and other anatomical sites that are difficult to reach with instruments and irrigants.

Another limitation concerns the choice of a mono-species biofilm model. Indeed, in natural biofilm communities *E. faecalis* is often found together with multiple microorganisms. However, in the current study the choice of a mono-species biofilm model was justified by the need to ensure simplicity and standardization of the protocol since no valid methods exist to manage each bacterial species within a multi-species biofilm due to their competitive interactions over time [5]. Despite single-species biofilm models are a better experimental method to investigate effectiveness of irrigants on *E. faecalis* eradication compared to planktonic bacteria, they still do not resemble real-life conditions, as these have a multispecies nature [1,28,58,59].

Additional limitations relies on the viability assessment. Culture methods has been widely used in assessing the number of viable and

cultivable bacteria in a sample by plating on agar plates [60] and quantifying CFUs. However, only viable bacteria, able to divide and form colonies, was quantified through this approach [61]. In root canal infections, a large amount of bacteria are viable but nonculturable (VBNC), meaning that despite their inability to grow in culture media, they are virulent, metabolically active and able to form biofilm, although to a lesser degree than viable bacteria [62].

As a matter of fact, the findings of this study have to be seen in light of some limitations as the culture methods for viability assessment which does not quantify VBNC bacteria, the use of paper point for sampling procedure and the choice of a mono-species biofilm model. Further experimental and clinical studies are necessary to better evaluate the effectiveness of new devices in the eradication of *E. faecalis* in canals with complex anatomy and to establish a more direct connection with clinical practice.

5. Conclusions

Within the limitations of the current study, it can be concluded that NaviTip and IrriFlex irrigation needles effectively removed *E. faecalis* demonstrated a high and comparable efficacy in removing mature *E faecalis* biofilms from curved canals.

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Periodontal pathogens in atheromatous plaques. A controlled clinical and laboratory trial

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Introduction

In the past decade a large amount of data suggested a possible relationship between periodontitis and cardiovascular disease 1-4). Several authors have reported significant associations between periodontal parameters (probing depth, clinical attachment level and tooth loss) and the extent and severity of the atherosclerosis (5). These data have gradually provided impetus to the infectious hypothesis of atherosclerosis, which suggests that localized infectious triggers may induce a chronic inflammatory response leading to the development and progression of atherosclerotic plaques (6). Many researchers focused their attention on a proposed ability of bacteria to colonize atheromatous plaques; several microorganisms, such as herpes virus (6), *Chlamydia pneumoniae* (7) and Cytomegalovirus (8), were identified in atheroma. More recently, Chiu (9), Haraszthy *et al*. (10) and Taylor-Robinson *et al*. (11) reported on the ability of periodontal pathogens to colonize atheromatous plaques in the carotid artery. Unfortunately, no information concerning the oral/periodontal condition and no data about periodontal infection were reported in these studies. The aims of this study were: (i) to ascertain the presence of periodontal bacteria DNA [*Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythensis* (formerly *Bacteroides forsythus*)] (12) in carotid atheromatous plaques in dentate and edentulous cardiovascular patients and (ii) to assess the concomitant presence of the same bacterial DNA, if any, in periodontal pockets and in carotid atheroma in the same dentate patient.

Material and methods

Study population

The study population consisted of consecutive patients scheduled for carotid endarterectomy at the Department of Vascular Surgery (University of Florence, Italy) during a 7-month period. The patients were identified as candidates for carotid endarterectomy according to the standard medical practices of the vascular surgery department. Patients who had undergone antibiotic treatment during the last 6 months and/or who had received periodontal or dental treatments in the last 6 months were excluded from the study. Informed consent was obtained from all the study participants before examination.

Fifty-two patients were enrolled in the study before hospital admission. The test group consisted of 26 dentate patients. The control group consisted of 26 patients edentulous for at least 2 years before examination. Information about smoking history, hypertension, diabetes and cardiovascular disease was obtained through clinical chartings and interviews. Oral and X-ray examinations (radiographic orthopanoramic) were performed on both the test and control patients. A complete periodontal examination (pocket depth, clinical attachment level, plaque index, bleeding on probing in six points/tooth) was performed in the test group by a single periodontist (CF). A sample of subgingival plaque was collected from the deepest pocket of each dentate patient. After a careful supragingival scaling, a sterile paper tip point was inserted in the pocket and maintained *in situ* for 10 s. Subsequently the paper tip point was inserted in an individual vial and sent to the laboratory. The surgical procedures for carotid endarterectomy were performed 3 weeks after the oral examinations. Atheromatous plaques were harvested from all patients during surgery. The specimens were placed in a vial with 10 ml of sterile saline solution, free of previous contaminating DNA, and frozen at −20°C. Both subgingival plaques samples and surgical specimens were sent to the laboratory (LAMMB, Department of Molecular Biology, University of Siena).

Laboratory procedure

Subgingival plaque samples and carotid specimens were analysed by the study microbiologist (GC). DNA was extracted from the subgingival plaque samples according to Contreras *et al*. (**13**). The transport vials containing the atheromatous specimens were opened only in the laminar air flow safety cabinet at the microbiology laboratory. All the specimens were kept at −20°C until processing. Care was taken to maintain aseptic handling of tissue samples. Approximately 100 mg of tissue was harvested from the middle of the atherosclerotic plaque and then homogenized and subjected to DNA extraction. A standard protocol for DNA extraction was used based on proteinase-K digestion and the use of cetyltrimethylammonium bromide (CTAB) to remove complex polysaccharides that may inhibit polymerase chain reaction (PCR) amplification. The extracted DNA was measured by spectroscopy. The amounts of DNA extracted ranged from 30 ng to 100 ng for each endarterectomy specimen. The human beta-globin test was used as the control for inhibiting the amplification (**14**).

Primer's design

Subgingival plaque and carotid specimens were examined using a PCR method for periodontal bacteria. The primers developed were characterized using the Oligo version 6 program (MedProbe, Oslo, Norway). The primers for *P. gingivalis* were designed on the collagenase gene sequence (GenBank Accession number **AB006973**). The primer **OG49** (AAGGACTGAATATGCGCGCCCGATC) was complementary to the sequence 131–155; the primer Og50 (GAGGTGATAATTCGCTCTCGG TCCCTACATCT) was complementary to the sequence 1275–1306. The annealing temperature was 60°C and the length of the predicted product of the first amplification was 1176 bp. The primers for *A. actinomycetemcomitans* were designed on the *glyA* gene and the *lKtC* gene (GenBank Accession number **Z23269**). The primer **OG57** (AGCGGACGTGAA AGAACTTGC) was designed using the *glyA* gene of strain **JP2** (GenBank Accession number **Z23269**) and was complementary to the sequence 1317–1337. **OG58** (GCAATAGGAA

CCCCATCTCTCAT) was designed using *lKtC* gene of the strain **JP2** (GenBank accession number **M27399**) complementary to the sequence 258–280. The primers developed to detect *P. intermedia***OG51** (GTGCTTGCAC ATTCTGGACGTCGAC) and OG-52 (CGTCTGCAATTCAAGCCCGGGTAAG), *F.nucleatum***OG41** (GGCCAC AAGGGGACTGAGACA) and **OG42** (TTTAGCCGTCA CTTCTTCTGTTGG), *T.forsythensis*, **OG45** (GTCGGACTAATACCTCAT AAAACA) and **OG46** (TCGCC CATTGACCAATATT) were designed using the 16S small subunit ribosomal RNA gene sequences. All of the primer sequences were compared with the GenBank to ensure their specificity.

Positive controls

P. gingivalis, *T. forsythensis, P. intermedia* and *F. nucleatum* gene targets were cloned into pGemT-easy plasmids (Promega, Milan, Italy) and used as positive controls. *A. actinomycetemcomitans* CCUG 37005 was cultured on Shaedler Anaerobe Agar (Boehringer Mannheim, Gmbh, Germany) incubated in a chamber for 7 days at 37°C in anaerobic atmosphere created with AnaeroGen System (Sigma, St Louis, MO, USA). Colonies obtained from cultures were suspended in 500 µl of sterile water. DNA was extracted and quantified both by spectrophotometer and electrophoresis and used as the positive control.

PCR conditions

The PCR protocol consisted of a preliminary denaturation step (95°C for 5 min), 35 cycles of annealing (annealing temperature of each pair of primers for 1 min), extension (72°C for 1 min and 40 s) and denaturation (95°C for 1 min) and a final elongation (5 min at 72°C).

Analysis of PCR products

A 10 µl aliquot of amplified samples from a PCR tube was electrophoresed through a 1% agarose gel (Sigma) for 30 min. After 20 min of ethidium bromide staining (0.5 µg/ml), the amplification products were visualized

and photographed under a UV light transilluminator (Bio-Rad Laboratories, Hercules, CA, USA).

Detection limit

In order to determine the PCR detection limit, plasmids were extracted from *Escherichia coli* by QIAprep Spin6 according to the manufacturer's instructions. Plasmid DNA was quantified by a spectrophotometer using 10-fold serial dilutions. Dilutions ranged from 10^8 to 10 copies/ml and 1 µl for each one was used in PCR to determine the sensitivity of the methods. DNA extracted from biological sample dilutions were spiked into beta-globin positive atheromatous DNA samples for the evaluation of the lower PCR detection limit.

Results

A total of 52 patients (26 dentate and 26 edentulous) were enrolled in the study. Twelve out of 52 patients (seven in test group and five in control group) were excluded because they were negative to DNA amplification using human beta-globin specific-primers. The test group (dentate patients) consisted of 19 patients (14 males and five females) with a mean age of 71.37 ± 6.14 years. Eleven patients were smokers. The mean tooth loss was 13.0 ± 6.25, mean clinical attachment level was 4.69 ± 1.58, mean pocket depth was 2.87 ± 0.82, mean full mouth plaque score was 75.95% ± 26.81 and mean full mouth bleeding score was 58.85% ± 27.15. The DNA of at least one of the probed bacteria was detected in each subgingival sample. The DNA of *T. forsythensis* was detected in 79% of patients, *F. nucleatum* in 63%, *P. intermedia* in 53%, *P. gingivalis* in 37% and *A. actinomycetemcomitans* in 5% of samples (Table 1). No statistically significant association between the periodontal pathogens was found. The control group (edentulous patients) comprised 21 patients (15 males and six females); the mean age was 73.33 ± 6.11. Anamnesis was generally positive for periodontal disease, as the patients reported that their teeth were frequently lost with elevated mobility (single patient results not shown). For

the edentulous patient group no PCR for oral pathogens was performed, as were no comparable sites for sampling.

Table 1. Results of polymerase chain reaction detection in subgingival plaque samples

Bacterial species	Frequency of detection
Tanarella forsythiensis	15/19 (79%)
Fusobacterium nucleatum	12/19 (63%)
Prevotella intermedia	9/19 (53%)
Porphyromonas gingivalis	8/19 (37%)
Actinobacillus actinomycetemcomitans	1/19 (5%)

The carotid specimens of all 40 patients revealed evidence of severe atherosclerosis; the plaques showed a calcified core with frequent circumferential fatty deposits. No DNA of periodontal bacteria was detected by PCR in any of the carotid samples in either patient group. The analysis of the detection limit of the PCR-based technique demonstrated the effectiveness of this procedure. Irrespective of the pathogen (*P. gingivalis*, *T. forsythensis*, *P. intermedia*, *F. nucleatum* and *A. actinomycetemcomitans*), the methodology allowed for the amplification of 10 genome equivalents. When using spiked clinical samples the detection limit was found to be 100 genome equivalents, which is equivalent to 1000–5000 bacteria per gram of tissue sample.

Discussion

Different studies have suggested a possible association between periodontitis and the extent and severity of cardiovascular diseases **1**-**5**). However, complete evidence has not been established in this field and other studies which questioned this association have been published **15**-**17**).

Experimental studies have demonstrated the ability of such periodontal pathogens to interact with the endothelial surface (**18**) and to induce smooth cell proliferation (**19**) and the local release of inflammatory cytokines (**20**). Therefore the presence of periodontal bacteria in human atherosclerotic plaques **9**-**11**) may play a role in the initiation, development and progression stage of atherosclerosis.

The present case-control study was first aimed to ascertain the presence of periodontal bacteria DNA in carotid atheroma in dentate and edentulous patients. Periodontal examination in the test group demonstrated a high rate of patients with chronic periodontitis associated with high mean age, poor oral hygiene and smoking history.A total of 52 carotid samples were collected and analysed for DNA of periodontal pathogens (*P. gingivalis*, *T. forsythensis*, *P. intermedia*, *F. nucleatum* and *A. actinomycetemcomitans*) by PCR. Twelve out of the 52 carotid samples were excluded from the analysis because they were negative to the human beta-globin test used as the control for inhibition of the DNA amplification. The reason for this may be explained by the fact that certain reagents such as porphyric components may hinder the results of the amplification (**21**).Subgingival plaques from 19 dentate patients (corresponding to dentate patients with carotid samples positive to human beta-globin test) revealed the presence of at least one of the periodontal species. No statistically significant association among pathogens was reported. This microbial profile reflects the findings described in specific studies that investigated the composition of subgingival plaque in much larger sample of patients (**22**, **23**). In neither the dentate nor edentulous patient groups was DNA of periodontal pathogen detected in the carotid atheromatous plaques.Our data do not concur with the results published by Chiu (**9**) (immunocytochemical investigation – 42% of

carotid plaques positive for *P. gingivalis*), Haraszthy *et al*. (**10**) (PCR-amplified 16S rDNA and DNA species-specific probes, 30% positive for *T. forsythensis*, 26% for *P. gingivalis*, 18% for *A. actinomycetemcomitans*, 14% for *P. intermedia*), Taylor-Robinson *et al*. (**11**) (PCR-amplified 16S rDNA and universal primers, 22% positive for *A. actinomycetemcomitans* and 9% for *P. intermedia*) and Ishiara *et al*. (**24**) (PCR-amplified 16S rRNA, 21.6% positive for *P. gingivalis*, 23.3% for *A. actinomycetemcomitans*, 5.9% for *T. forsythensis*). These studies identify at least one, but often multiple, periodontal pathogen in atheromatous samples, whereas we have no evidence of periodontal bacteria DNA in our sample collection.A possible reason for this difference could be the methodology used in different laboratories. PCR procedures may extensively vary for extraction procedures, primers designs and reaction conditions. The primers used in this PCR study differ from those applied in the previous studies. The primers for *P. gingivalis* and *A. actinomycetemcomitans* were designed using specific gene sequences; those for *T. forsythensis*, *P. intermedia* and *F. nucleatum* were developed using the 16S small subunit ribosomal RNA gene sequences. The efficiency of our primers was confirmed on clinical samples spiked with plasmid DNA. An alternative hypothesis, even if less probable, could be that the prevalence of periodontal bacteria DNA in atheromatous lesions differs due to epidemiological reasons (disease stage, nutrition, geographic factors, ethnicity, etc.). However, the presence of bacterial DNA in atheromatous plaques still remains a controversial issue. An example is a multicenter PCR comparison trial for the detection of *C. pneumoniae* in endarterectomy specimens, in which the positivity rate varied between 0 and 60% using different test methods on the same atherosclerotic plaques (**25**).In conclusion, the results of this study do not support the previous findings that reported a frequent presence of periodontal pathogens in carotid atheroma lesions. Our data, therefore, tend to exclude a direct correlation between the detection of periodontal bacteria DNA in oral lesions and its concomitant presence in carotid atheroma.

8

8.0 Adherence to Mediterranean Diet, Physical Activity level and severity of periodontitis. Results from a University-based cross-sectional study.

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Introduction

Unhealthy lifestyle behaviours are at the root of the global burden of noncommunicable diseases (NCDs), which account for about 63% of all deaths (Marrero et al., 2012). Unhealthy lifestyle behaviours include poor nutrition, physical inactivity, poor sleep quality, high stress, and tobacco/alcohol use (Kushner & Sorensen, 2013). Each of these lifestyle behaviours constitute the multiple determinants of "health" as defined by the US Department of Health and Human Services (US Department of Health and Human Services). Over the past several years, there has been an increased interest in evaluating the benefit of adhering to healthier lifestyle behaviours in the development of morbidity and mortality (Kushner & Sorensen, 2013). In many epidemiological studies, patterns of healthy lifestyle behaviours, characterized by high diet quality (sometimes referred to as high adherence to Mediterranean Diet - MD), adequate frequency of physical activity (PA), and not smoking, were associated with a lower risk of NCDs onset, cardiovascular events, and mortality when compared to subjects with unhealthy lifestyles (Chiuve et al., 2011; Ford et al., 2009; Stringhini et al.,

2010). The detrimental impact of unhealthy lifestyles on systemic health may be mainly ascribed to the induction of a state of low-grade systemic inflammation (LGSI) and to the overproduction of reactive oxygen species (ROS), leading to oxidative stress (Esposito et al., 2004; Frodermann et al., 2019). Since LGSI has been bi-directionally linked with periodontitis, the association between lifestyle behaviours and periodontitis has been analyzed in several epidemiological studies, which reported a higher prevalence as well as a more severe disease phenotype in subjects with unhealthy lifestyle behaviours (Coelho et al., 2020; Karaaslan & Dikilitaş, 2019; Marruganti et al., 2022; Romandini et al., 2017; Marruganti et al. Under review).

Indeed, unhealthy lifestyles may also negatively influence the efficacy of the Steps 1 and 2 of periodontal therapy through the molecular pathways of LGSI imbalance and ROS overproduction. However, the longitudinal impact of lifestyle behaviours on the clinical periodontal outcomes after Steps 1/2 has not been evaluated yet. Therefore, the aim of the current pre-post quasi-experimental study was to assess the longitudinal impact of lifestyle behaviours on the efficacy of the Steps 1/2.

Materials and methods

Source of data

The current pre-post quasi-experimental study is reported according to the TRIPOD (Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis) statement guidelines (Moons et al., 2015). The research protocol was approved by the local ethics committee (protocol

number: 19037/2021) and was registered on Clinicaltrials.gov (NCT04769622).

Participants

Participants were selected among those included in a previous study (Marruganti et al., 2022; Marruganti et al., Under review) according to the following inclusion criteria:

-presence of untreated Stage II/III periodontitis (Tonetti et al., 2018) with at least one site with Probing Pocket Depth (PPD) ≥ 4 mm;

-presence of at least 20 remaining teeth;

-age between 18 and 70 years old;

-ability and willingness to give informed consent.

The exclusion criteria were:

-current pregnancy or lactation;

-periodontal therapy received in the previous 12 months;

-antibiotics intake within the previous 6 months;

-inability to effectively communicate in Italian.

Individuals were enrolled in the study after they read and signed the written informed consent, in accordance with the Declaration of Helsinki.

Assessment of study variables

Lifestyle behaviours

At BL, lifestyle behaviours were assessed by administering a set of four validated questionnaires: i) adherence to Mediterranean Diet questionnaire (QueMD) (Gnagnarella et al., 2018); ii) International Physical Activity

Questionnaire (IPAQ) (Mannocci et al., 2010); iii) Perceived Stress Score (PSS) questionnaire (Cohen et al., 1983); iv) Pittsburgh Sleep Quality Index (PSQI) questionnaire (Mondo et al., 2021). According to the sum scores obtained in each questionnaire, participants were categorized as having either high vs. low adherence to MD, moderate/high vs. low PA level, low vs. moderate/high perceived stress, and good vs. poor sleep quality. Further details regarding the structure of the questionnaires and their sum scores assessment methods are reported in the Appendix 1.

Clinical periodontal variables

At baseline (BL), all included participants received a full-mouth periodontal examination by two blinded and previously calibrated examiners (C.M., C.G.; see Appendix 2 for the calibration process). PPD, recession (REC), plaque (O'Leary et al., 1972) and bleeding on probing (BoP) (Ainamo & Bay, 1975) were recorded with a standardized periodontal probe (UNC 15 probe, HuFriedy Group, Chicago, Illinois, United States) at six sites per tooth, third molars excluded. Clinical attachment levels (CAL) were computed as the sum between PPD and REC values. Tooth mobility was measured according to the Miller classes (Miller & Boenheim, 1938). Clinical periodontal variables were recorded again at re-evaluation 3 months (3M) after the completion of Steps 1/2.

Periodontal therapy

After the completion of the questionnaires, all participants received Steps 1/2 of periodontal therapy by two blinded therapists (C.M., C.G.). Periodontal

therapy was performed following a stepwise approach, which included: i) oral hygiene instructions (OHIs) and motivation for successful domiciliary plaque control and risk factors management, as well as supragingival Professional Mechanical Plaque Removal (PMPR) performed with ultrasonic instruments (Cavitron® Select SPS, Dentsply Sirona, Rome, Italy) (Step 1), and ii) quadrant-wise subgingival instrumentation performed with both ultrasonic and hand instruments (Gracey curetters, HuFriedy, Chicago, USA) under local anesthesia (Articaine 4% with epinephrine 1:100.000) (Sanz et al., 2020) (Step 2). One month after the completion of the Steps 1/2, participants received a reinforcement of OHIs and motivation.

Outcomes

The primary endpoints of Steps 1/2 were: i) endpoint of therapy (binary), i.e., absence of sites with PPD ≥ 4mm with BOP, and of sites with PPD ≥ 6mm; and ii) pocket closure (continuous), i.e., the proportion of sites with PPD ≤ 4mm at 3M. At patient-level, other outcomes encompassed: FMPS, FMBS, n PPD ≥ 4mm, n PPD ≥ 6mm, and n teeth with mobility ≥ 1. At site-level, PPD/REC/CAL changes between 3M and BL (3M - BL) were considered as endpoints.

Confounders

Self-reported socio-demographic characteristics were registered, together with information regarding the presence of any comorbidity with potential effects on periodontitis, and smoking status. The Body Mass Index (BMI) was computed as weight (kilograms)/height (meters^2). The detailed

assessment methods of socio-demographic characteristics are reported in the Appendix 1.

Statistical analyses

Analyses were performed using a statistical software (STATA BE, version 17, StataCorp LP, Texas, United States), *a priori* setting the level of significance at 5%. Continuous variables were reported as Mean and Standard Deviation (SD); categorical data were expressed as number of observations (percentage-%).

After verification of data distribution, comparisons of periodontal variables between BL and 3M were performed using the paired Student's t-test. Simple and multiple linear/logistic regression analyses were performed to investigate the relationship between lifestyle behaviours and each periodontal parameter (3M - BL) both at site- and patient-level. Multilevel regression analysis was applied for site-level data (sites clustered at patient-level). The following multiple models were considered: i) Model 1: estimates adjusted for the value of the variable at baseline (e.g., outcome "PPD change" adjusted for the PPD value at BL); ii) Model 2: Model 1 + smoking + comorbidities (binary variable, which equalled 1 whenever the participant had least one of the recorded comorbidities); iii) Model 3: Model 2 + FMPS at 3M + FMBS at 3M. Results from regressions analyses were expressed as odds ratios (ORs) or difference in means (MD) with 95% CI. The binary variable "unhealthy lifestyles" (which equalled 1 whenever each participant had: low adherence to MD AND low PA level AND high perceived stress AND poor sleep quality, otherwise it equalled 0) was built and was regarded as the main

predictor of the final multiple prediction models. Each model was built to assess the predictive ability of the main predictor (independent variable "unhealthy lifestyles") for the outcomes of Steps 1/2 at 3 months (outcome variables at site- and patient-level). The predictors included in each model encompassed: variable at BL, age, Gender, BMI, occupation, education, smoking status, comorbidities, and brushing frequency. Each outcome variable and the predictors were analyzed through the `allsets` command, and the best prediction model for each outcome was chosen according to the lowest value of the Mallow's Cp statistic for linear models, and according to the highest value of Area Under the Curve (AUC), and the lowest values of Akaike (AIC) and Bayesian (BIC) information criteria for logistic models. The predictors selected for the inclusion in each best prediction model are listed for each outcome in the Tables' footnotes.

Results

From the 235 participants included in a previous investigation (Marruganti et al., 2022; Marruganti et al., Under review), a total of 120 participants was eligible for inclusion in the present study. The participants' selection process is detailed in Figure S1. At 3M, one subject was lost to follow up (n=119). Most of the participants were males (58.3%) with a mean age of 58.8 years. Around 55% of participants had low MD adherence, 42.5% performed low PA, 34.2% had moderate/high PSS, and 39.2% had poor sleep quality (Table 1). At BL, participants had a mean FMPS of 73.2%, with a mean number of sites with PPD ≥ 4mm of 28.7 (±16.7) (Table 2).

Efficacy of Steps 1/2

Overall, Steps 1/2 achieved a mean pocket closure of 61.9%, with 76 (63.9%) participants achieving the endpoint of therapy at 3M. Steps 1/2 led to a significant reduction of FMPS and FMBS, as well as a significant reduction in the number of sites with PPD ≥ 4mm and PPD ≥ 6mm (p<0.001). The mean number of teeth with mobility almost halved from BL to 3M (Table 2).

Efficacy of Steps 1/2 *by subgroups of lifestyle behaviours*

At site-level, simple and multiple models demonstrated a significantly lower PPD change in subjects with low aMed/low PA (vs. high aMed/high PA, respectively); although the same trend was highlighted also for the changes in the other periodontal variables, the estimates were significant only for CAL change in Model 1/Model 2 (p<0.05). Both moderate/high PSS and poor sleep quality were associated with significantly lower REC and CAL changes in the simple and multiple regression models (Model 1), while PPD change was significant in Model 1 only for moderate/high PSS, but not for poor sleep quality (Table 3).

At patient-level, a non-significant tendency toward worse periodontal variables in low aMed/low PA subjects at 3M was highlighted; the estimates were significant for low PA subjects, which showed significantly lower proportions of pocket closure in the simple and multiple models (Model 3: MD=-15.3; 95% CI: -27.4, -3.3; p<0.05). The same trend for pocket closure was followed by the moderate/high PSS category (Model 3: MD: -16.9; 95% CI: -29.7, -4.1; p<0.05). Moreover, poor sleep quality showed an inverse

association with the endpoint of therapy in both the simple and multiple models (Model 3: OR=0.2; 95% CI: 0.07, 0.6; $p<0.01$) (Table 4).

Prediction models

Results of the multiple prediction models at site- and patient-level are shown in Table 5 and Table 6. At site-level, the variable "unhealthy lifestyles" significantly predicted lower changes in PPD, REC, and CAL at 3M ($p<0.01$) (Table 5). At patient-level, the combination of "unhealthy lifestyles" predicted the presence of a significantly lower number of sites with PPD ≥ 4mm when compared to subjects with healthier lifestyles (MD=14.5; 95% CI: 7.6, 21.4; $p<0.001$); the same trend was highlighted for pocket closure (MD=-19.3; 95% CI: -35.8, -2.9; $p=0.022$) (Table 6).

Discussion

Main findings

The present pre-post quasi-experimental study demonstrated a significant independent relationship between unhealthy lifestyles, such as low MD adherence, lack of physical exercise, high perceived stress, and poor sleep quality, and lower reductions of periodontal variables at site-level 3 months after Steps 1/2 of periodontal therapy. Similarly, patient-level periodontal variables at 3M were tendentially worse in subjects with poor lifestyle behaviours. The combination of low MD adherence, physical inactivity, high stress, and poor sleep quality ("unhealthy lifestyles") resulted as a significant predictor for site- and patient-level outcomes of Steps 1/2. At 3M, the combination of "unhealthy lifestyles" tended to have more residual

periodontal pockets and a lower proportion of closed pockets than subjects with healthier lifestyles.

Interpretation

In the current investigation, Steps 1/2 led to a pocket closure of around 62%, which is consistent with previous meta-analytical data showing a proportion of 57% of pockets closed at 3-4 months follow up (Suvan et al., 2020). Nonetheless, the values of pocket closure achieved in the current study varied widely across subgroups of lifestyle behaviours (between 69.8% and 53.6%), with lower proportions noted for subjects assuming unhealthy lifestyles behaviours. These differences were consistent and significant also after adjusting for baseline values, inflammatory variables (plaque and bleeding at 3M), as well as other factors (i.e., comorbidities and smoking status) that could have otherwise influenced the subject's response to Steps 1/2 (Hsu et al., 2019; Suvan et al., 2020). Hence, an impact of lifestyle behaviours on periodontal treatment response can be hypothesized. Longitudinal studies investigating the influence of different lifestyle behaviours on the outcomes and efficacy of Steps 1/2 of periodontal therapy are currently lacking. Conversely, plenty of epidemiological reports highlighted a cross-sectional association between periodontitis occurrence and severity with unhealthy lifestyle behaviours, namely low MD adherence, low PA, high stress, and poor sleep quality (Coelho et al., 2020; Karaaslan & Dikilitaş, 2019; Marruganti et al., 2022). Overall, these unhealthy lifestyle habits lead to an increased state LGSI through a variety of molecular pathways (Besedovsky et al., 2019; Esposito et al., 2004; Frodermann et al., 2019; Sabbah et al.,

2018). In particular, a low MD adherence dietary pattern is usually characterized by the frequent consumption of pro-inflammatory foods, such as white flour and processed meats, which also contribute to increasing oxidative stress levels (Christ et al., 2019). Similar molecular pathways leading to an imbalance of LGSI and an overproduction of ROS were also involved in the association between low PA (or sedentary lifestyle), poor sleep quality and periodontitis, respectively (Besedovsky et al., 2019; Frodermann et al., 2019). In addition to these mechanisms, high stress was also found to exert an immunosuppressive action on the immune system (e.g., by reducing lymphocyte proliferation and antibody production) and to trigger other behavioural adaptive changes (e.g., alcohol/drug use, increased smoking, etc.) (Sabbah et al., 2018). Since it has been previously shown that these molecular pathways can negatively influence the periodontium and render the subjects more prone to periodontitis onset and rapid progression (D'Aiuto et al., 2010; Pink et al., 2015), it can be hypothesized that the same mechanisms can reduce the efficacy of Steps 1/2, as observed in the current investigation.

In the last few years, evidence flourished regarding how lifestyle behaviours (e.g., diet, PA, stress, sleep quality, etc.) should not be considered as separate entities but, instead they should be regarded in a more holistic way. Indeed, the latest consensus on the Mediterranean diet pyramid incorporated the suggested frequency, portions, and types of foods with other recommendations, such as getting enough sleep, and performing regular physical exercise, in order to achieve a healthy and balanced lifestyle (Bach-

Faig et al., 2011). Hence, the effects of each lifestyle behaviour add up and thus positively or negatively contribute to the subject's oral and systemic health (Bach-Faig et al., 2011; Furman et al., 2019; Lambrinou et al., 2019). The current study also investigated the impact, as well as the predictive ability, of the combination of unhealthy lifestyles, i.e., low MD adherence, low PA, high stress, and poor sleep quality, on the outcomes of the Steps 1/2 of periodontal therapy. Results derived from the prediction models obtained showed that a combination of specific unhealthy lifestyles predicts a lower performance of periodontal treatment at 3 months with an adequate predictive ability.

Implications

Results derived from the prediction models of the current study highlighted that unhealthy lifestyle behaviours not only impact on periodontitis onset and severity, but also influence the efficacy and the clinical outcomes of the Steps 1/2. Such prediction models could help the clinician identify at baseline a high-risk subgroup of patients who are more likely to achieve nonoptimal clinical outcomes after the Steps 1/2. Hence, for those patients, clinicians could be able to plan customized lifestyle interventions targeted at the amelioration of specific lifestyle dimensions (i.e., diet, sleep, stress, physical activity). As also indicated by the EFP S3 level guidelines, behavioural changes interventions could be implemented both by educating or advising the patients, or in some cases by referring the patient to a specialist (Sanz et al., 2020). A possible downside to the implementation of lifestyle interventions may be some added costs for the patient/clinician. Although the

current study did not run a cost-benefit evaluation, the systemic health benefits that can be obtained from these interventions if they are successful would represent reduced costs for the prevention and management of other comorbidities (e.g., diabetes, hypertension) (Lambrinou et al., 2019; Ramseier & Suvan, 2015; Sanz et al., 2020; Sun et al., 2017).

The current findings should be regarded with caution considering the limitations of this study. First of all, the selected sample was not representative of the study population and therefore there is a lack of external validity, as well as a risk of residual confounding due to the observational study design. In addition, the presence of information bias could not be excluded given that lifestyle behaviours were recorded using questionnaires which, although validated, represent self-reported information. Furthermore, the validity of the present findings only applies for a short follow up period (3 months).

Conclusion

The present pre-post quasi-experimental study showed that subjects following unhealthy lifestyles, such as low MD adherence, lack of physical exercise, high perceived stress, and poor sleep quality, achieved worse clinical periodontal outcomes at both site- and patient-level 3 months after Steps 1/2 of periodontal therapy, when compared to subjects with healthier lifestyles. In addition, the combination of the four mentioned "unhealthy lifestyles" at baseline can predict a lower performance of the Steps 1/2 at 3-month follow up. Randomized controlled trials are warranted to elucidate the

periodontal response to the implementation of the Step 1 phase of periodontal treatment with customized lifestyle interventions for each patient.

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Conclusion and future directions

The aim of the endodontic treatment is to treat or avoid, by reducing intracanal bacterial load beneath the immune system activation, the apical periodontitis (1). The presence of bacteria in sessile form in the root canal apical third, seems to be the main etiological factor of AP both in primary and post-treatment infections showing that this kind of infection could be considered part of biofilm mediated infections (2). While it was profoundly demonstrated the role of resident microbiota as community of unity of pathogenicity (3), *Enterococcus faecalis* is the most prevalent bacterial species found in post-treatment apical periodontitis with relative abundance that could reach the 100% of total bacteria (4). The persistence of bacteria at the time of filling is the main cause of upsurge of endodontic infections even though no specific species was linked to it (5), while in some cases they may be secondary invaders resulting by coronal microleakage or contamination during the previous treatment (6). It is widely accepted that the first source of root canal contamination is represented by the communication created by the caries forefront while many studies declared doubts about the role of bacteria present in the caries process in contribution of endodontic infection (7). In the light of this results, many researcher sights on the saliva as main responsible of endodontic space contamination (8). In our study we demonstrated that, taking *Enterococcus faecalis* as bacterial model, the main source of secondary endodontic infections was the saliva using, for the first time, a combination of culture microbiological test and the newest Dna assay like the whole genomic technique. The results of this study bring to light, from a clinical point of view, the need to stress the adequate positioning of the rubber dame and to avoid any external contamination from operative field.

Based on this fact the microbiological goal of endodontic treatment is to reduce as much as possible the bacterial load at the end of shaping and finishing procedures and, by the combined use of guttapercha and sealers providing the entombing of the residual bacterial cells in order to avoid its multiplication and any contact with immune system cells. For the first purpose we tested for a reduction of *E.faecalis* contamination with a new endodontic needle in curved canals. While our study doesn't show any differences with other needles present on the market, the prolipropilene needle show more flexibility respect to the others, reaching the working length also in difficult anatomic conditions.

In the last decades many antimicrobials were developed for endodontic use to get better results in terms of both bacteriostatic and bactericidal activity. Between those, antimicrobial peptides have caught the attention of the researchers. In our study we tested two new antimicrobial peptides in an ex vivo model against *Enterococcus faecalis* clinical strain resistant to the conventional chemomechanical procedures. The results showed the efficacy of both the peptides tested in the inhibition of biofilm formation and could represent a new way in fighting biofilm related diseases. At the same time the sealing capability of our endodontic treatment play a key role in maintenance of periapical health. The choice of the sealers, to use together to guttapercha when closing the endodontic space, could play a key role in the long-term stability and in the same way could prevent the recolonization of the remaining bacteria in root canal system. In the recent years, the market has opened to new calcium silicate-based sealers. Their physico-chemical properties and alleged antimicrobial activities, together to a very easy clinical use by single cone technique, have produced a large consensus about the clinicians all the world. In our study we tested, for five physicochemical properties, a new bioceramic sealer compared to a conventional zinc-eugenol sealer demonstrating that all the standards were respected except for setting time in case of test sealer.

The resolution of pre-existent peri-apical lesions could be affected by systemic conditions jeopardizing the post-treatment healing processes (9). Periapical lesion is un inflammatory process regarding the tissue around the

apex in most of the times triggered by a microbial infection of the root canal space. Recently many studies tried to associate oral infections to systemic comorbidities demonstrating that, in case of periodontal diseases, the increasing of systemic inflammatory mediators could have consequences on general health. Considering the similarity in term of inflammatory response, it may be possible that AP have the same consequences (10). Recently new published data showed an increment of inflammatory mediators of systemic burden in patient with apical periodontitis. Regarding this issue, it is well documented how incorrect lifestyle could increase the plasma levels of proinflammatory cytokines affecting the quality and the metabolism of bone tissue (11). We developed an observational study in which the aim of the study was evaluating the influence of adherence to Mediterranean diet, sleep disorders, physical activity and stress on DMFT score, presence of periapical lesion and its gravity. Interestingly we found a very clear association between lifestyles and periapical lesion that were linearly correlated to PAI score index.

Recently, a new meta-analysis study, conducted on the global burden of apical periodontitis, showed that 52% of analyzed population had this infection with spike reaching 63% when a systemic comorbidity was present (12). In front of this data, the present Ph.D thesis showed attention on local and systemic factors that could influence the presence of peri-apical lesions in primary infections and its persistence after endodontic treatment. From a clinical point of view would be necessary to avoid any external contamination during endodontic treatment especially in patients with compromised systemic conditions. Further research activities needed to improve endodontic irrigation protocols and to better understand how systemic conditions could affect the physiopathology of peri-apical lesions.

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